



BIOAUGMENTATION FOR REMEDIATION OF CHLORINATED SOLVENTS:

TECHNOLOGY DEVELOPMENT, STATUS, AND RESEARCH NEEDS

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FOREWORD

Bioaugmentation involves the introduction of microorganisms into contaminated media to promote the degradation of contaminants. Though viewed with skepticism in the past, the use of bioaugmentation has increased significantly in recent years, with mounting evidence that it can be helpful for improving the bioremediation of some contaminants, under some site conditions. Use of bioaugmentation has become particularly popular for increasing the rate and extent of reductive dechlorination of chlorinated solvents.

Because of the increasing use of this technology, the Environmental Security Technology Certification Program (ESTCP) commissioned this White Paper on bioaugmentation to review the state of the science at the present time. Most of the work on this White Paper was done by staff at GeoSyntec, though other vendors and experts have contributed as well.

The objectives of this White Paper are to: 1) summarize the current status of this rapidly-evolving innovative technology; 2) identify the key issues confronting the science; and 3) evaluate the lessons learned from current practical applications. In particular, we hope that this review will be useful to remedial project managers faced with selecting, designing and implementing a bioaugmentation strategy.

Any review of an emerging technology can only hope to capture a snapshot in time. This White Paper is intended to summarize the publications and experiences prior to the May 2005 Battelle conference on In Situ and On-Site Remediation. It is our hope that this White Paper can be expanded and updated in coming years as more information on the subject becomes available.

This White Paper cannot provide definitive guidance on the selection and implementation of bioaugmentation. There remains considerable controversy about whether bioaugmentation is necessary, how it should be implemented at specific sites, and how cultures for bioaugmentation should be handled and applied to the subsurface. While this White Paper identifies these controversies and provides some perspective on the issues involved, it is too early to develop definitive conclusions.

The practice of bioremediation is difficult to assess because there are few peer-reviewed publications available. Therefore, it has been necessary to rely heavily on the so-called “gray literature” and on the information and experiences supplied by vendors practicing in this area, particularly the experiences of the primary authors. Such an approach has inevitable limitations and can result in a perception of bias. This White Paper was reviewed by other bioaugmentation vendors and outside experts in the area, in an attempt to minimize the potential for bias and to ensure that the information presented is as complete and technically accurate as possible. Nevertheless, some speculation and subjectivity are unavoidable given the current state of the science. In addition, the level of detail is limited by the fact that many vendors may be licensing organisms or techniques that they consider privileged and confidential. This White Paper is intended to stimulate more peer-reviewed research to answer the questions raised in this review.

Any such review will also tend to focus on the reported successes, and there have clearly been several successful applications. Well-monitored field demonstrations have shown unequivocally that added dechlorinating cultures can become established in the saturated zone, and

bioaugmentation can lead to faster and more complete dechlorination of chlorinated ethenes. Not all bioaugmentation projects have been clear-cut successes, but it is hard to find information regarding perceived failures. Even for some of the apparent successes, bioaugmentation may not have been essential for effective treatment, given sufficient time. But again, it is hard to find well-documented cases in which the costs and benefits of bioaugmentation have been evaluated in side-by-side comparisons.

Bioaugmentation has a checkered history. In the early years of bioremediation, several vendors sold cultures that had little or no value under field conditions. Although there have been several successful demonstrations in recent years, it remains a controversial and insufficiently understood technology. This White Paper will not resolve all of the controversy or answer all of the questions regarding bioaugmentation. ESTCP's hope is that this White Paper will contribute to a broader understanding of the advantages and limitations of bioaugmentation, identify the key data gaps and outstanding issues, and eventually lead to useful guidance and protocols for the selection and application of this technology.

Dr. Jeffrey Marqusee
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EXECUTIVE SUMMARY

The intent of this White Paper is to summarize the current technical and regulatory status of bioaugmentation, and to identify research needs to be addressed in upcoming years to facilitate widespread successful use of the technology. The focus of the White Paper is on bioaugmentation for chlorinated ethenes, as this is a critical need for the Department of Defense (DoD) and because of the advanced application and commercialization of bioaugmentation cultures for these compounds.

Research in the early 1980s provided the first evidence that common chlorinated solvents such as tetrachloroethene (PCE), trichloroethene (TCE), 1,1,1-trichloroethane (1,1,1-TCA) and carbon tetrachloride (CT) could be biodegraded by microorganisms that are naturally present in soil and groundwater. However, until the recent development of rapid and inexpensive molecular techniques for microbial identification, little was known regarding the specific microorganisms mediating these biodegradation reactions. As a result, an entire bioremediation industry developed for the remediation of chlorinated solvents in soil and groundwater without a comprehensive understanding of the relevant microbiological processes involved, including the identities of the bacteria mediating the degradation reactions, their nutrient requirements, and the appropriate methods for stimulating the desired degradation reactions while minimizing competitive or undesirable microbial activities.

In the late 1980s and early 1990s, the growing acceptance of bioremediation to treat petroleum hydrocarbons and wood preserving wastes led to a proliferation of vendors offering microbial inoculants for bioaugmentation. However, experience showed that many of these inocula were not effective under field conditions, and the process of bioaugmentation fell into disrepute. Now, after more than 10 years of laboratory and field research in the area of chlorinated solvent bioremediation, the process of bioaugmentation is once again the subject of significant debate. With the recent recognition that the microorganisms that completely biodegrade chlorinated ethenes such as PCE and TCE to ethene (a non-toxic product) are not ubiquitous in all subsurface environments, or may not be present in sufficient numbers to degrade the contaminants at acceptable rates, there is mounting evidence that bioaugmentation can improve the bioremediation of chlorinated ethenes in soil and groundwater.

So why is bioaugmentation in 2005 different than in 1992? First, a number of microorganisms have now been identified that are capable of utilizing chlorinated ethenes as electron acceptors (dehalorespiration). Further, it is apparent that some members of the genus *Dehalococcoides* are capable of metabolic reduction of dichloroethenes and vinyl chloride to ethene. *Dehalococcoides* microorganisms have been detected at field sites where complete dechlorination has been observed, and are these microbes are present in all commercially-available bioaugmentation cultures.

Several enriched microbial cultures have been described in the peer-reviewed literature and developed for commercial use. These include KB-1TM (developed at the University of Toronto and commercialized by SiRem), the Bachman Road culture (the source for both Regenesi's BioDechlor INOCULUMTM and the BC2 inoculum marketed by BioAug LLC), and the Pinellas culture (developed by GE and licensed to Terra Systems). In addition, there are a number of other cultures currently being used in research and commercial applications, including SDC-9

developed by Shaw Environmental, Inc., and several unnamed cultures marketed by Bioremediation Consulting Inc. Bioaugmentation has been successfully used with a wide variety of electron donors, including lactic acid, vegetable oil, molasses, and HRCTM.

Most of the major vendors stressed the need to maintain explicit quality control procedures to ensure continued technical credibility. The QA/QC procedures that should be used include regular testing of the culture's capability to degrade the target contaminants, testing and operational practices designed to ensure that known pathogens are not part of the cultures, and testing of the numbers of dechlorinators and the levels of dechlorinating activity on a regular basis. In addition, guidance and protocols are needed for the introduction and monitoring of bioaugmentation cultures in the subsurface.

The most obvious application of bioaugmentation is at sites that completely lack the requisite *Dehalococcoides* microorganisms or where complete dechlorination of PCE and TCE does not occur. At sites with an indigenous *Dehalococcoides* population, bioaugmentation may still provide significant benefits, including the introduction of a *Dehalococcoides* strain possessing superior dechlorination properties and reductions in the necessary acclimation periods at sites with inadequate numbers of *Dehalococcoides*. Based on the recognition that dechlorinating microorganisms are capable of activity at high chlorinated ethene concentrations, the application of bioaugmentation in source areas containing either PCE or TCE as dense, non-aqueous phase liquids (DNAPLs) is an emerging area of technology application.

Remedial project managers (RPMs) considering bioaugmentation at specific sites must decide not only whether to bioaugment, but also when and how to do so. The evidence to date suggests that bioaugmentation is essential at a relatively small fraction of sites. However, it can be helpful in reducing the time required for complete dechlorination to occur at a much larger number of sites. This reduced time for acclimation can be economically attractive by reducing the overall O&M costs, and it can also be valuable in increasing the confidence by regulators and the public that the remedy is in fact effective.

Bioaugmentation can be performed at the start of treatment (soon after reducing conditions have been established), as a sort of insurance, or as is often the case, it can be reserved as a contingency in case complete dechlorination is not observed. This decision will depend on the costs for bioaugmentation as well as the regulatory and political environment. Technically, there is some concern that bioaugmentation will be less effective if reserved as a contingency, because it will become increasingly difficult for the added organisms to become established in the subsurface over time. However, there is not enough experience to date to understand if this concern is legitimate.

The economics of bioaugmentation depend on the amount of culture needed, the source of the inoculum, and on the need for recirculation to foster distribution of the culture throughout the target zone. Active recirculation has been used in most demonstrations, but the costs for recirculation can make bioaugmentation more expensive than simply operating a biostimulation system for several extra months, or even years. However, there is increasing evidence that relatively low-cost, "passive" bioaugmentation (direct injection of culture solutions, without recirculation) can be effective at many sites.

Within existing regulatory frameworks, bioaugmentation is most commonly regulated within the Underground Injection Control permit process. Although this is a federally-mandated program, in many states, jurisdictional authority is ceded to state legislatures that have promulgated an equivalent regulatory process. To date, bioaugmentation has been approved for use in at least 21 states.

Although it is too early to provide definitive guidance on bioremediation, several practical conclusions can be drawn from this review:

1. Several cultures are commercially available, and their value has been demonstrated under field conditions.
2. Cultures can be grown efficiently, transported to field sites effectively, successfully injected, and in most cases they will survive and grow in aquifers given proper environmental conditions.
3. RPMs should address the issue as early in the design stage as possible, and perform an explicit cost-benefit assessment, including a life-cycle cost analysis, to determine whether bioaugmentation has the potential to reduce the time and costs for bioremediation.
4. The costs for bioaugmentation generally represent a low fraction of the total remediation costs (typically 1-3%). In many cases, passive bioaugmentation can pay for itself if it reduces the time needed for complete dechlorination by even a couple months.
5. The key practical issues appear to be determining *a priori* whether bioaugmentation will be beneficial, ensuring adequate distribution of added cultures throughout a target zone, overcoming potential inhibitory conditions, and ensuring adequate quality controls.
6. The roles of the other organisms present within the mixed cultures used for bioaugmentation are not clear. Other organisms appear to be needed for complete dechlorination, at least at some sites.
7. Site-specific tests to characterize the indigenous microbial population have been developed, and can be useful in deciding whether to bioaugment. These tests include targeted microbiological and molecular biological analyses that can rapidly assess the potential for complete dechlorination.
8. RPMs should work closely with the culture vendors to ensure that the cultures are added in a manner that maximizes the potential for success. The timing and locations of injections, and the numbers of organisms added, should all be carefully designed in a cooperative manner.
9. The methods used to add and distribute the augmentation cultures are key economic considerations. Most vendors report considerable success with much less expensive

“passive” injection techniques, although definitive demonstrations of this approach are not yet available.

Key environmental factors (notably redox, pH, and concentrations of organic carbon and electron acceptors) must be monitored and controlled before, during, and after injections, to ensure that the added organisms have the best possible opportunity to survive and thrive.

1. INTRODUCTION

Research in the early 1980s provided the first evidence that common chlorinated solvents such as tetrachloroethene (PCE), trichloroethene (TCE), 1,1,1-trichloroethane (1,1,1-TCA) and carbon tetrachloride (CT) could be biodegraded by microorganisms that are naturally present in soil and groundwater (*e.g.*, McCarty *et al.*, 1981; Bouwer and McCarty, 1983; Vogel and McCarty, 1985; Fogel *et al.*, 1986; Wilson and Wilson, 1985; Freedman and Gossett, 1989). This research focused primarily on identifying the rates and products of biodegradation under varying environmental conditions.

Until the recent isolation of the responsible bacteria and the development of rapid and inexpensive techniques for microbial identification, little was known regarding the specific bacteria mediating these biodegradation reactions. As such, an entire bioremediation industry developed for the remediation of chlorinated solvents in soil and groundwater with an incomplete understanding of the relevant microbiological processes involved, including the identities of the bacteria involved, their nutrient requirements, and the appropriate methodologies for stimulating the desired degradation reactions while minimizing competitive or undesirable microbial activity.

In retrospect, it is evident that this early research had two major impacts on the subsequent development of the bioremediation industry. First, a surprising number of vendors began to develop and market microbial inoculants in the 1980s, claiming the ability to biodegrade a wide range of compounds, including chlorinated solvents, petroleum hydrocarbons, polynuclear aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and chlorinated phenols (Major and Cox, 1992). These inoculants typically contained aerobic and facultative microorganisms commonly found in soil and groundwater. The application of these various inocula yielded mixed results and brought to light the difficulties of bioremediation chlorinated solvent compounds.

The vendor claims for many of these microbial inoculants often exceeded their actual performance, generating significant skepticism regarding the reliability of bioaugmentation, particularly for chlorinated solvent bioremediation. By 1995, it was generally accepted that these products might be useful in some applications, such as for the bioremediation of chlorophenols or PAHs, but that they were likely to have limited impact on more recalcitrant contaminants such as chlorinated solvents. The second result of this early research was the expectation that robust and complete biodegradation of chlorinated solvents could be achieved by biostimulation alone (*i.e.*, by providing the indigenous microbial population with an excess supply of the appropriate electron donors or electron acceptors and any needed nutrients). This view presumed that the indigenous microbial communities at all sites included microorganisms capable of degrading chlorinated solvents. The conventional wisdom was that if the correct environmental conditions were created, the desired degradation reaction would invariably occur. To a large extent, this belief is still held by some bioremediation vendors who advocate the use of proprietary nutrient products (*e.g.*, electron donors) or patented bioremediation approaches (*e.g.*, Nyer *et al.*, 2003).

With the advent of molecular techniques for microbial identification, the bioremediation community, including the academic, consulting, industrial, and government sectors, is beginning to understand the role of microbiology in the success and failure of bioremediation under wide-ranging environmental conditions. This understanding has in turn led to the development and commercialization of several new microbial inoculants for the remediation of chlorinated solvents in groundwater, raising a fundamental question: what is the difference between these inoculants and those inoculants previously found to be ineffective?

First, the niche is relatively unique. Many organisms can degrade petroleum hydrocarbons, for example, and acclimation times are generally short. In contrast, bioaugmentation for chlorinated solvent remediation can require extremely long lag times in some cases, and the added organisms have shown an ability to become established following the development of sufficiently reducing conditions. Secondly, most of the new inoculants have been developed based on credible peer-reviewed research that is available in the public domain. Independent research by multiple organizations has provided a comprehensive understanding of the significant biodegradation pathways and the roles of the specific microorganisms that mediate these pathways.

In addition, independent field verification of culture performance is available for several of the commercially-available cultures, and should be forthcoming for some of the newer commercial products. Data from several field demonstrations of bioaugmentation have been reported in peer-reviewed journals (*e.g.*, Ellis *et al.*, 2000; Major *et al.*, 2002; Lendvay *et al.*, 2003), and these reports have greatly increased confidence in the general approach and in the reliability of bioaugmentation as a credible technology. Finally, improved testing methodologies are now available to determine whether the bacteria known to be responsible for specific chlorinated solvent biodegradation reactions are present at a site, providing the ability to predict (to some extent) *a priori* whether bioaugmentation may be required at a given site.

The intent of this White Paper is to summarize the current technical and regulatory status of bioaugmentation as a supporting tool for bioremediation of chlorinated solvents, and to identify the research needed to facilitate the successful use of the technology. The focus of the White Paper is bioaugmentation for chlorinated ethene remediation, as this is a critical need for the Department of Defense (DoD) and because of the advanced application and commercialization of bioaugmentation cultures for these compounds. The following sections of this White Paper specifically provide:

- An historical perspective of bioaugmentation, focusing on the early uses (and misuses) of the technology in the 1980s and early 1990s (Section 2);
- A discussion of the scientific research conducted in the 1990s through 2004 to understand the microbiology involved in the degradation processes (Section 3);
- A discussion of the microbiology of dehalorespiration, which is the most common chlorinated solvent biodegradation mechanism used by bioremediation practitioners today, and the biodegradation mechanism promoted by the commercially-available microbial cultures (Section 4);
- A summary of the current state of knowledge surrounding the microbial cultures that are currently commercially available (Section 5);

- A summary of select bioaugmentation field demonstrations (Section 6);
- Information regarding the regulatory permit requirements for bioaugmentation field demonstrations that have been conducted (Section 7); and
- A description of information and research needs to be addressed to improve technical and stakeholder acceptance of this technology, and to optimize the benefit of this technology for use by government and industry (Section 8).

The first drafts of this White Paper were originally prepared by Mr. Evan Cox and Drs. Eric Hood, David Major, Mary DeFlaun and Neal Durant of GeoSyntec Consultants, and Mr. Philip Dennis of SiREM, with assistance from Dr. Elizabeth Edwards of the University of Toronto, and Dr. James Gossett of Cornell University, as part of an ESTCP-funded project by GeoSyntec, to demonstrate and improve the state of the art of bioaugmentation (<http://www.estcp.org/projects/cleanup/CU-0315.cfm>).

The ESTCP Program Office then sought independent reviews from other bioaugmentation vendors and experts, and edited the document based on those comments. In addition, several other experts in the area have reviewed the draft documents and have made significant contributions to the final document, in an effort to minimize the potential for bias and to ensure that the information presented is as complete and technically accurate as possible. These reviewers and contributors include Dr. Stephen Koenigsberg and Ms. Erin Rasch of Regenesys, Dr. Michael Lee of Terra Systems, Drs. Sam Fogel and Margaret Findlay of Bioremediation Consulting, Inc., Dr. Rob Steffan of Shaw Environmental, Mr. Robert Steele of BioAug LLC, and Dr. Frank Loeffler of Georgia Tech University

2. EARLY DEVELOPMENT OF BIOAUGMENTATION

In the late 1980s and early 1990s, the growing acceptance of bioremediation to treat petroleum hydrocarbons and wood preserving wastes led to a proliferation of vendors offering microbial inoculants for groundwater and soil bioremediation. For example, in a survey of microbial inoculants for bioremediation published by the Ontario Ministry of the Environment (MOE) in 1992, seventy-five commercial inoculants for *in situ* bioremediation were identified, of which about 10% claimed the ability to treat, among other things, halogenated aliphatic compounds (Major and Cox, 1992).

Although some inoculants were proprietary and others not characterized, the survey indicated that most inoculants were composed of common soil microorganisms (*e.g.*, various species of *Pseudomonas*, *Bacillus*, *Mycobacterium*, *Micrococcus*, *Phanaerochaetes*, *Alcaligenes*, *Nocardia*, *Thiobacillus*, *Arthrobacter*, *Flavobacterium*) grown under aerobic conditions. The minimal characterization of the microbial constituents of these cultures, coupled with often exaggerated claims regarding efficacy against a broad range of contaminants (as shown in Table 1), indicate the minimal degree of performance testing and validation considered acceptable by the industry at that time.

Vendors that disclosed the contents of their inoculants stated that they were not attempting to provide a ready-made microbial population that would immediately degrade target contaminants; rather, the apparent benefits of their products were the convenience and time-savings associated with:

- Efficiencies derived by optimizing their inoculant through the intentional culturing and blending of different bacterial species; and
- Establishing high population densities of the appropriate microbes, which was anticipated to lead to rapid contaminant degradation.

It was often difficult to assess the applicability and effectiveness of the available inocula, because the biodegradation pathways for many of the subject chemicals were not understood or documented at the time. At a minimum, researchers were skeptical of vendor claims that these added microbes could out-compete the indigenous organisms, and added microorganisms often failed to become established because of a lack of understanding of the ecological factors controlling the subsurface microbial population (*e.g.*, Goldstein et al., 1985). The prevailing ecological theory was that microbial species present at a site were best suited to their niche, making the natural communities stable even when subjected to moderate levels of biotic or abiotic stress (Sulflita, 1989). Furthermore, the general consensus in the early 1990s was that the genetic potential to degrade most if not all contaminants already existed in the environment, which could be expressed by manipulation of environmental conditions.

The failure of these inoculants to improve the rate or disappearance of fuel hydrocarbons may have been due to their inability to compete with indigenous microorganisms (Lee and Levy, 1987; Venosa *et al.*, 1996), or simply that they did not add additional metabolic potential to that

Table 1: Characterization of Commercial Bioaugmentation Inocula (Circa. 1992)

Company	Identification	Chemicals Treated	Source of Inoculum
Alko Biotechnology Ltd. ¹	Mixed culture	Oil Contaminated Solids	Enrichment culture from contaminated soil
Alpine Remediation Products (distributor for Reliance Biochemical Corporation) ¹	Unidentified mixed culture (RBC 212)	Gasoline, Diesel, Benzene, Toluene, Xylenes, Ethyl Benzene and Polyaromatic Hydrocarbons	Proprietary
	Unidentified mixed culture (RBC 216)	Petroleum Oil & Grease, Animal Fats & Oils, Triglycerides and Polyaromatic Hydrocarbons	Proprietary
	Unidentified mixed culture (RBC 218)	Heavy Petroleum Hydrocarbons, Phenol, Polyaromatic Hydrocarbons, Creosote, Pentachlorophenol Wastes	Proprietary
Argonne National Laboratories ²	Mixed culture	Diesel Fuel	Isolate from contaminated site
Bioscience, Inc. ³	Mixed culture of <i>Pseudomonas</i> , <i>Nocardia</i> , <i>Bacillus</i> , <i>Micrococcus</i> , etc. under tradename Microcat	Petroleum Hydrocarbons, Solvents, Monomers, Pesticides	In-house supplier
BioTrol, Inc. ¹	<i>Flavobacterium spp.</i>	Pentachlorophenol, Polyaromatic Hydrocarbons	In-house culture collections, ATCC
	<i>Methylosinus trichosporum OB3b</i>	Chlorinated Solvents (Trichloroethene)	In-house culture collections, ATCC
EMCON Associates ¹	Mixed culture <i>Bacillus spp.</i>	Petroleum Hydrocarbons	Isolates from soil
Enviroflow, Inc. ¹	<i>Bacillus subtilis spp.</i>	Petroleum Hydrocarbons, Animal and Vegetable Fats, Proteins, Carbohydrates Creosote, Phenols, Municipal Sewage	Commercial supplier
ESE Biosciences, Inc. ¹	Mixed culture predominantly composed of <i>Pseudomonas</i> , <i>Micrococcus</i> , <i>Corynebacterium</i> , <i>Mycobacterium</i> and <i>Actinomycetes</i>	Petroleum Hydrocarbons, Phenols, Phthalates, Solvents, Municipal Wastes	Proprietary
Heritage Remediation Engineering ²	Mixed culture (proprietary mixtures of identified microorganisms)	Alcohols, Aromatics, Carbohydrates, Detergents, Ketones, Petroleum Hydrocarbons, Phenols, Phthalates, Solvents, Municipal Wastes	Contaminated site isolates
	Mixed culture (proprietary mixtures of identified microorganisms)	Methyl Ethyl Ketone, Methyl Isobutyl Ketone, Toluene	Municipal sewage treatment plant
	Mixed culture (proprietary mixtures of identified microorganisms)	Acrylic Acid	Industrial biological treatment plant
		Diesel and Gasoline	Municipal sewage treatment plant

**Table 1: Characterization of Commercial Bioaugmentation Inocula (Circa. 1992)
continued)**

Company	Identification	Chemicals Treated	Source of Inoculum
Kiseki Inc. ¹	Unidentified mixed culture (KBC 101, 102, 107, 109)	Polychlorinated Biphenyls (PCBs), Pentachlorophenols, Hydrogen Sulfide (H ₂ S), Phenols, Foams, Detergents, Oil & Grease, Cresol	Isolated from areas yielding hydrocarbon degraders-oil spills, salt lakes
	Unidentified mixed culture (KBC 100)	Hydrocarbons	
Merkert Laboratories Inc. ¹	Unidentified (ML-21)	Hydrocarbons, Diesel Fuel Pentachlorophenols, Jet Fuel, Toluene, Ethylene Glycol, all organics except Polychlorinated Biphenyls	Commercial collection
	Unidentified (Bio-degreaser)	Fats, Oils, Greases	Commercial collection
Osprey Biotechnics ³ (culture currently provided by CL-Solutions)	<i>Pseudomonas fluorescens</i>	Anthracene, Benzene, o-Chlorotoluene, p-Chlorotoluene, Cresols (mixed), Dinitrooctylphthalate, Dichlorobenzene, 1,1-Dichloroethane, 1,2-Dichloroethane, Dichloropropane, Dichlorotoluene, Ethylene Glycol, Pentachlorophenol, Phenanthrene, Phenol, 1,1,2-Trichloroethane	Proprietary
Osprey Biotechnics ³ (continued)	<i>Pseudomonas putida</i>	m-Chlorotoluene, Crude Oil, 2-Ethoxyethanol, Ethylene Glycol, Ethylbenzene, Isoprenoids, Methyl Ethyl Ketone, Methylene Chloride, Naphthalene, Terpene, 1,1,2,2-Tetrachloroethane, Toluene, Waste Oils/Sludges, Xylene	Proprietary
Polybac Corporation ¹	Polybac products include: <i>Acinetobacter calcoaceticus</i> , <i>Aspergillus oryzae</i> , <i>Bacillus cereus</i> , <i>Bacillus megaterium</i> ,	Animal and Wastes, Fibrous Wastes, Filamentous Organisms, Fats, Oils and Greases, Oil Field and Refinery	Proprietary

**Table 1: Characterization of Commercial Bioaugmentation Inocula (Circa. 1992)
continued)**

Company	Identification	Chemicals Treated	Source of Inoculum
Polybac Corporation ¹ (cont.)	<i>Bacillus subtilis</i> , <i>Bacillus thuringiensis</i> , <i>Micrococcus sp.</i> , <i>Myxobacter</i> , <i>Nitrobacter sp.</i> , <i>Nitrosomonas sp.</i> , <i>Saccharomyces cerevisiae</i> , <i>Thiobacillus</i>	Wastes, Cellulose, Lignins, Pulp and Paper Wastes, Ammonia, Hydrocarbons in Saline Environments, Hydrocarbons, Phenols, Polyaromatic Hydrocarbons, Aromatics, Glycols, Formaldehyde, Waxes, Cyanide, Proteins, Carbohydrates	
ReTeC, Remediation Technologies, Inc. ²	<i>Phanerochaete chrysosporina</i> <i>Pseudomonas spp.</i> Anaerobic Mixed culture <i>Pseudomonas spp.</i> <i>Alcaligenes spp.</i> <i>Pseudomonas spp.</i> Mixed culture	Polyaromatic Hydrocarbons Pentachlorophenol DDT Polyaromatic Hydrocarbons 2,4-D, 2,4,5-T 2,4-D, 2,4,5-T Cycloalkanes, Oils	Utah State University Culture collection, in-house treatment site Culture collection, in-house treatment site Culture collection, in-house treatment site Culture collection, in-house treatment site University collection Culture collection, in-house treatment site
SBP Technologies, Inc. ¹	<i>Pseudomonas putida</i> <i>Pseudomonas paucimobilis</i> <i>Pseudomonas spp.</i> <i>Pseudomonas cepacia</i>	Creosote, Polyaromatic Hydrocarbons, Fluoranthene, Polyaromatic Hydrocarbons Pentachlorophenol Trichloroethene	Soil sludge samples from contaminated sites Soil sludge samples from contaminated sites Soil sludge samples from contaminated sites TCE-contaminated sites
Technical Resources, Inc. ¹	<i>Pseudomonas cepacia</i>	Trichloroethene, Dichloroethene Toluene, Phenol, o-, m-, p-Cresol, o-, m-, p-Xylene, Styrene, Naphthalene, Cumene, Indole, Anthranilic Acid	Laboratory stock culture isolated from industrial waste treatment lagoon
Waste Stream Technology, Inc. ³	<i>Pseudomonas putida</i> <i>Alcaligenes faecalis</i> <i>Pseudomonas spp.</i> (fluorescent)	Polyaromatics, Total Petroleum Hydrocarbons, Halogenated Organics, Fuel Oils, Jet Fuel Aromatics (BTEX), Gasoline, Volatile Organic Compounds (VOCs), Phenols Jet Fuel, Diesel, Fuel Oil, Waste Oil, Phthalate Esters, Cutting Oil, PAHs	Site isolate Site isolate Site isolate

**Table 1: Characterization of Commercial Bioaugmentation Inocula (Circa. 1992)
continued)**

Company	Identification	Chemicals Treated	Source of Inoculum
Waste Stream Technology, Inc. ³ (cont.)	<i>Pseudomonas spp.</i>	Aromatics (BTEX), Volatile Organic Compounds, Gasoline, Phenols, Pentachlorophenol	Site isolate
	<i>Arthrobacter spp.</i>	Pentachlorophenol	Site isolate
	<i>Pseudomonas fluorescens</i>	Crude Oil, Fuel Oil, Diesel Fuel	Site isolate
	<i>Pseudomonas fluorescens</i>	Crude Oil	Marine isolate
	<i>Acinetobacter johnsonii</i>	Crude Oil, Fuel Oil	Marine isolate
	<i>genospecies 7</i> <i>Psychrobacter spp.</i>	Crude Oil, Diesel, Fuel Oil	Site isolate

(after Major and Cox, 1992)

Notes

BTEX - benzene, toluene, ethylebenzene, xylene

VOC - volatile organic compound

PAH - polycyclic aromatic hydrocarbons

TCE - trichloroethene

¹ No contact information available

² Company no longer provides culture as a commercial product

³ Company currently provides culture as a commercial product

already present within the soil. Numerous studies have conclusively demonstrated that populations of oil-degrading bacteria in soil and water increase in the presence of oil (Button *et al.*, 1992; Lee and Levy, 1987; Prince 1993; Atlas, 1993).

Furthermore, field trials have shown that the addition of either commercial inoculants (Lee and Levy, 1987) or enriched cultures of indigenous hydrocarbon degrading bacteria (Fayad *et al.*, 1992; Venosa *et al.*, 1996) did not significantly enhance the biodegradation rates of oil when compared to the addition of nitrogen- and phosphorous-based nutrients (Atlas and Bartha, 1972; Prince, 1994; Swannell and Head, 1994). These studies showed that the metabolic potential to degrade petroleum hydrocarbons is near-ubiquitous in the environment.

The Ontario MOE report generally concluded that acceptance of microbial inoculants for soil and groundwater remediation would require:

- Proof that bioaugmentation for a specific contaminant is necessary. Specifically, confirmation that the required degradation activity is absent from the indigenous microbial population;
- Proof that the inoculants contain live, healthy bacteria with the ability to completely degrade the target contaminants, and that such bacteria can successfully compete with indigenous bacteria;
- Proof that inoculants can be distributed in the soil and groundwater given the effect of adsorption, nutrient competition, filtration and predation;
- Confirmation from replicated field trials with proper controls and appropriate monitoring that any observed contaminant degradation results from the bioaugmented microbial populations;
- Implementation of efficacy and quality controls to ensure a consistent composition of the microbial inoculant between production batches;
- Verification that inoculants are free of plant and animal pathogens (particularly those inoculants composed of a consortia of microorganisms);
- Low cost, requiring only small amounts of added biomass, and not requiring extreme manipulations of environmental conditions to allow introduced organisms to be effective; and
- Prevention of specious claims by vendors.

The early development of bioaugmentation technology for *in situ* remediation may also have been hampered by the common misperception that bioaugmentation necessarily involved the release of genetically engineered microorganisms (GEMs) into the environment. Indeed, the emergence of new genetic engineering tools in the early 1990s led to the development of a variety of GEMs with specialized capabilities for biodegrading recalcitrant compounds (*e.g.*, Lajoie *et al.*, 1994; Pipke *et al.*, 1992; Krumme *et al.*, 1994). While most treatability tests using GEMs were performed in the laboratory, a limited number of tests were also performed at the field-scale. A lack of understanding regarding the risks of GEMs to human health ultimately led to public and regulatory opposition to bioaugmentation using GEMs. Through association with

GEMs and unrealistic vendor claims, early developments in the area of bioaugmentation were met with skepticism, and the process of bioaugmentation for site remediation was essentially dormant until the late 1990s.

3. RECENT PROGRESS IN CHLORINATED SOLVENT BIOREMEDIATION

Through persistent laboratory and field research over the past decade, a significant body of scientific literature has been amassed regarding the mechanisms and microorganisms involved in the biodegradation of chlorinated ethenes, ethanes, and methanes. The rapid growth in this field, in part spurred by the advent of improved microbial characterization techniques, has prompted the emergence of bioaugmentation for chlorinated ethene remediation. This section briefly chronicles the main research developments that have occurred in the area of chlorinated solvent biodegradation, leading to the current state of the practice, which is largely focused on the use of dehalorespiring microbial cultures to dechlorinate chlorinated solvents to environmentally-acceptable, non-chlorinated end products. Although in practice anaerobic dechlorination is the current focus of chlorinated solvent remediation, future research may discover other degradative mechanisms that will allow for a superior remediation technology to be developed.

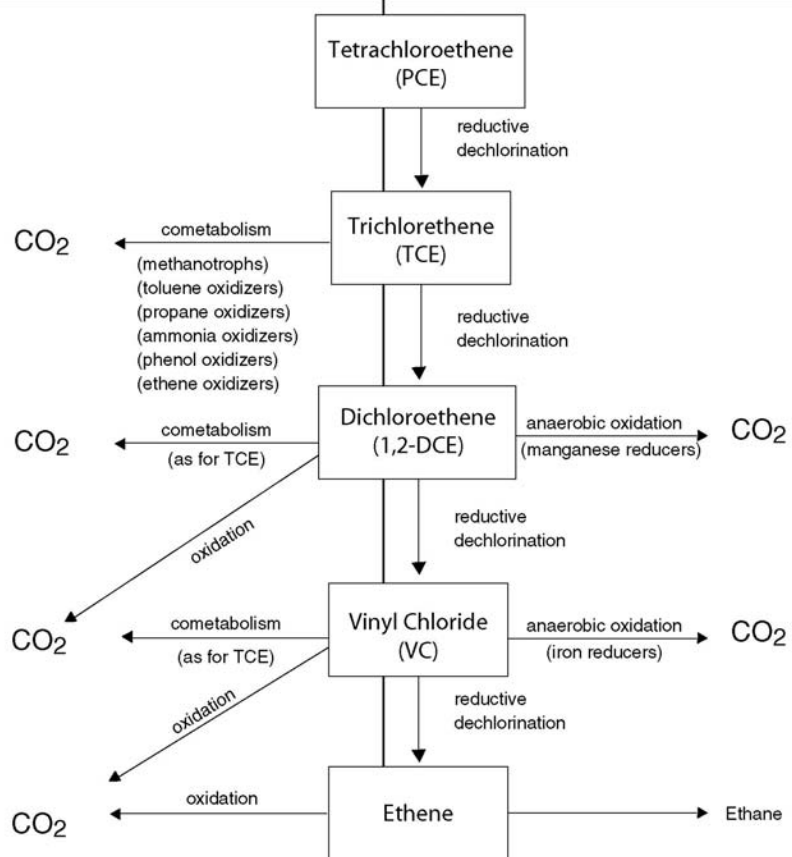
Figures 1a to 1c provide degradation pathways, including a few key references for each pathway, for common chlorinated ethenes, chlorinated ethanes and chlorinated methanes that are present in groundwater at DoD sites. Comparison of the degradation pathway references to literature describing the identification of the responsible organisms shows that the degradation pathways were typically identified years before the isolation and identification of the microorganisms involved. Through the 1990s, the identity of the microorganisms involved in these biodegradation mechanisms was often seen as less important than the functional expression of their degradation activity and the specific environmental conditions needed to promote that activity. This is consistent with the consensus viewpoint of the bioremediation industry at that time, which held the prevailing view that all that was required to effect the desired degradation reaction was manipulation of the geochemical conditions, which would in turn stimulate the microorganism(s) with the requisite metabolic pathways to degrade the target contaminant(s). Once the pathway was established, only then was the microorganism isolated and studied to better understand its metabolism.

Reductive dechlorination of PCE/TCE was recognized as early as 1983 (Bouwer and McCarty, 1983), but the research demonstrated that each subsequent reductive dechlorination step was slower than the proceeding one, resulting in the accumulation of vinyl chloride (VC). As a result, researchers temporarily abandoned the idea of applying anaerobic biodegradation of PCE/TCE due to the accumulation of VC, a compound more toxic and carcinogenic than the parent compounds.

At the same time (early 1980's), it was also discovered that chlorinated ethenes could be co-oxidized by aerobic bacteria. Cometabolism (co-oxidation) is the fortuitous transformation of a compound by an enzyme synthesized by the cell for metabolism of another compound. The chlorinated ethenes can degrade via cometabolic dechlorination (e.g., Fathepure *et al.*, 1987), although it is generally held that PCE is not amenable to cometabolic degradation, despite documentation of PCE cometabolism by *Pseudomonas putida* OX1 by Ryoo *et al.* (2000). Other organisms, including *Pseudomonas putida* and *Methylosinus trichosporium* OB3b, degrade

Aerobic Conditions

Anaerobic Conditions



Pathways for the Degradation of Chlorinated Ethenes

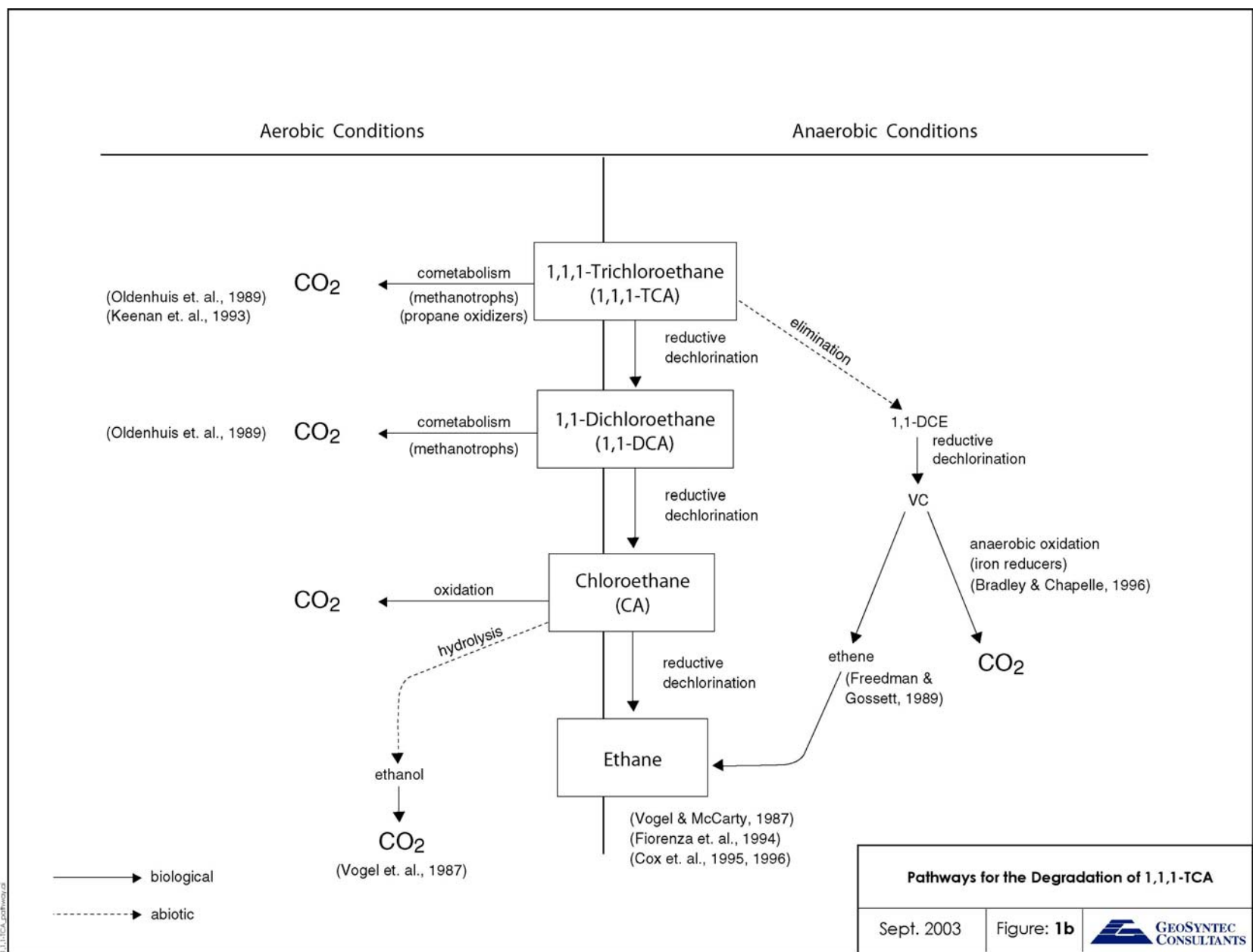
Kelly Air Force Base, San Antonio, Texas

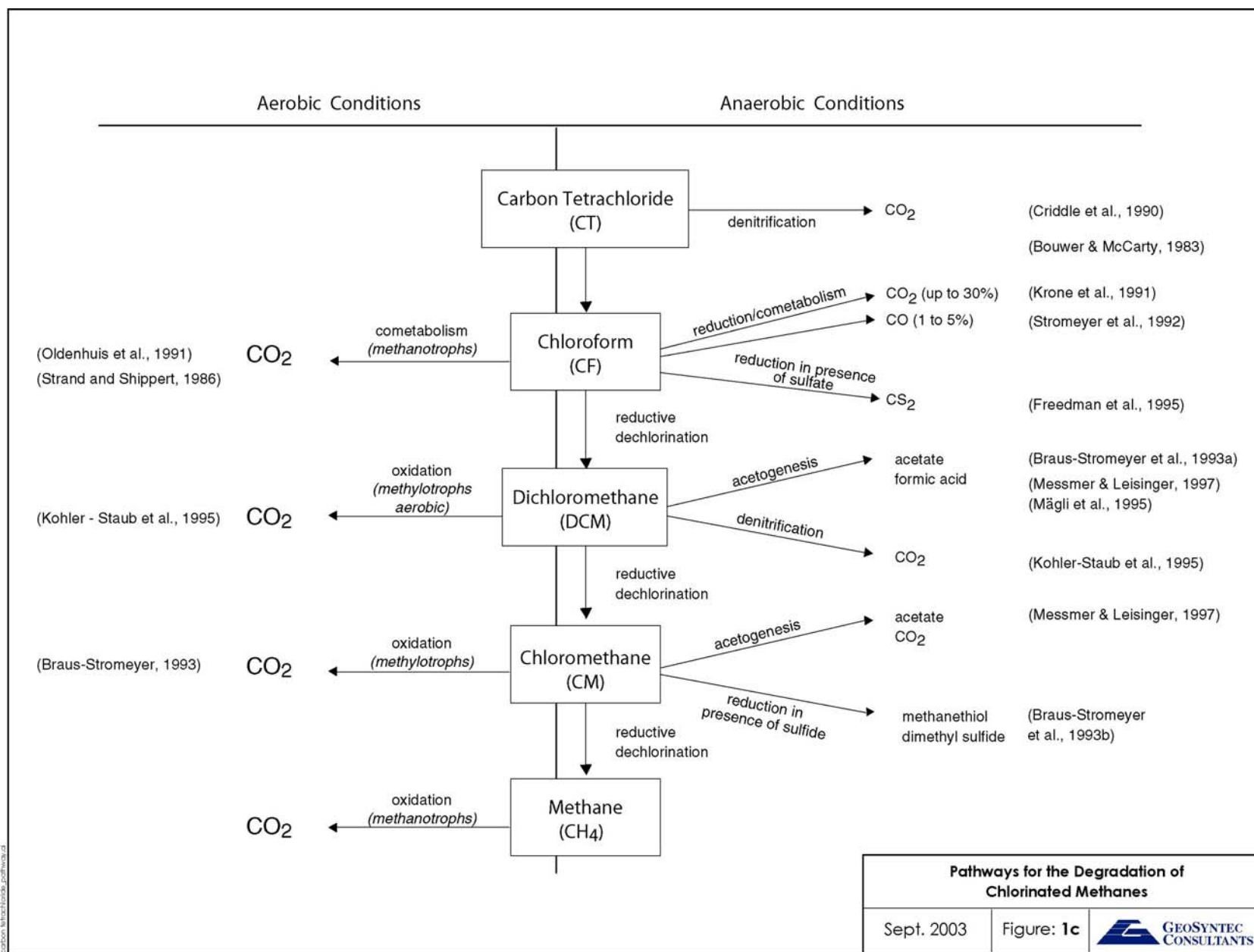
Jan. 2001

Figure: 1



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additional chlorinated compounds (*e.g.*, chloroethenes, chloroethanes, chloromethanes, and chloropropanes) via cometabolism (Heald and Jenkins, 1994; Oldenhuis *et al.*, 1989).

Initially, cometabolic degradation was viewed as an attractive process because: 1) it avoided the production of degradation intermediates such as VC; 2) it involved aerobic microorganisms which generate large amounts of biomass (*i.e.*, high yield) and are easier to handle in the field; 3) degradation rates were very fast (*i.e.*, half-lives of hours); and 4) many different types of organisms were found to harbor mono- and di-oxygenase enzymes with broad substrate specificity and the ability to co-oxidize chlorinated solvents. These benefits spurred significant research into the cometabolic degradation processes. Through this work, researchers found that aerobic co-metabolism could be induced through the addition of methane (Fogel *et al.*, 1986; Little *et al.*, 1988; Oldenhuis *et al.*, 1989; Fox *et al.*, 1990;), aromatic hydrocarbons (Nelson *et al.*, 1986; Wackett and Gibson, 1988; Winter *et al.*, 1989), ammonia (Arciero *et al.*, 1989), and propane (Wackett *et al.*, 1989).

Unfortunately, despite the promise of the aerobic cometabolism approach, field implementation was found to be very challenging and was met with a series of incremental setbacks. First, introducing enough oxygen and co-substrate proved to be difficult and/or expensive to implement (Steffan *et al.*, 1999). This led to the development of cultures using selected or genetically-engineered microorganisms that would constitutively (*i.e.*, would not require induction by a metabolite) express these enzymes (*e.g.*, Munakata-Marr *et al.*, 1996). However, adhesion of the introduced bacteria in the zone immediately surrounding the injection point limited the distribution of the microorganisms and the success of the bioremediation process for field-scale applications. To overcome this technical hurdle, both adhesion-deficient strains and ultramicrobacteria were developed (Cusack *et al.*, 1992; DeFlaun *et al.*, 1999) that possessed the desired degradation capabilities. However, after many years of field trials, aerobic cometabolism was determined to be too difficult for many to implement and sustain at most sites (relative to enhancing reductive dechlorination), and the approach has generally fallen out of favor for the remediation of chlorinated solvents.

While research on the aerobic biodegradation of chlorinated ethenes dominated much of the 1990s, some researchers continued to work on anaerobic reductive dechlorination, eventually demonstrating that VC could be further reduced to ethene (Freedman and Gossett, 1989), a break-through that changed the landscape of bioremediation of chlorinated solvents. Recognition that complete dechlorination was achievable, even at concentrations near the solubility limit for the most highly chlorinated compounds that do not degrade aerobically (DiStefano *et al.*, 1991) led to a renewed interest in this process and the microbiology of reductive dechlorination, as discussed in the following sections.

4. DEHALORESPIRATION: THE KEY PROCESS UNDERLYING CURRENT BIOAUGMENTATION PRACTICES

Over the last decade, scientists have discovered that several anaerobic bacteria can metabolically couple reductive dehalogenation to ATP synthesis in the cell, and thus obtain energy for growth from the energy released from the exergonic dechlorination reaction (Mohn and Tiedje, 1990; McCarty, 1994; Holliger *et al.*, 1998). In this process, chlorinated compounds act as terminal electron acceptors in metabolism, much like oxygen, nitrate or sulfate does for other organisms. Table 2 lists various anaerobic microorganisms that can use PCE and TCE as electron acceptors via dehalorespiration. During dehalorespiration, chlorinated ethenes undergo a process called reductive dechlorination, which results in the step-wise replacement of the individual chlorine atoms with hydrogen. An electron donor is required to provide energy for this process (McCarty, 1994). The direct electron donor for reductive dechlorination is often molecular hydrogen, which is typically produced in groundwater environments by the anaerobic oxidation (fermentation) of carbon substrates, such as organic acids or alcohols (Maymo-Gatell *et al.*, 1997).

4.1 The Ubiquity Concept Revisited

When the scientific community demonstrated that PCE and TCE could be completely dechlorinated through *cis*-1,2-dichloroethene (*cis*-DCE) and VC to ethene in both the laboratory (Freedman and Gossett, 1989) and the field (Major *et al.*, 1991), the expectation was that eventually many different types of organisms capable of complete dechlorination of PCE and TCE to ethene would be identified. However, after more than a decade of intense laboratory and field research, it appears that this expectation is likely incorrect.

There is a growing body of evidence that the specific microorganisms required to achieve complete dechlorination are not ubiquitous in the environment. “Stalling” at DCE has been observed at several sites, and evidence suggests that at least at some of these sites, the lack (or very low numbers) of competent bacteria is responsible. Of course, there are other possible reasons for an accumulation of DCE, including: 1) DCE is almost 4 times more soluble than TCE and can “emerge” and be retained in ways that would simulate a build-up related to poor metabolic response in the aquifer; and 2) that high levels of bioavailable iron and conversions from ferric to ferrous forms can interfere with electron flow to DCE (*e.g.*, Evans and Koenigsberg, 2001; Koenigsberg *et al.*, 2002; Koenigsberg *et al.*, 2003).

However, the non-ubiquity concept has considerable support, at least in some situations. For example, only microorganisms belonging to the genus *Dehalococcoides* have demonstrated the capacity to dechlorinate *cis*-DCE and VC to ethene (*e.g.*, Maymo-Gatell *et al.*, 1997), and, while *Dehalococcoides* do appear to be widespread, they do not appear to be ubiquitous (*e.g.*, Hendrickson *et al.*, 2002, Fennell *et al.*, 2001). Compelling evidence for the critical role of *Dehalococcoides* in chlorinated ethane bioremediation was provided by Hendrickson *et al.* (2002), who conducted a survey of the occurrence of this microorganism at 24 contaminated

Table 2: Summary of Dehalogenating Bacteria Detected in Pure and Mixed Cultures

Organism	GenBank Accession No. ¹	Taxonomic Affiliation	Known Electron Acceptors	Reference
Organisms Isolated in Pure Culture				
<i>Desulfomonile tiedjei</i>	M26635	<i>Proteobacteria</i> (subdivision)	3-chlorobenzoate	Townsend et al. (1997)
<i>Clostridium bifermentans</i> strain DPH-1	Y18787	Gram positive; <i>Clostridia</i>	PCE, TCE	Chang et al. (2000)
<i>Dehalobacter restrictus</i>	U84497	Gram positive; <i>Clostridia</i>	PCE, TCE	Holliger et al. (1993)
<i>Sulfurospirillum</i> (<i>Dehalospirillum</i>) <i>multivorans</i>	X82931	<i>Proteobacteria</i> (subdivision); <i>Campylobacteraceae</i>	PCE, TCE	Scholz-Muramatsu et al. (1995)
<i>Desulfotobacterium</i> strain PCE1	not available	Gram positive; <i>Clostridia</i>	PCE	Gerritse et al. (1996)
<i>Desulfotobacterium</i> sp. strain PCE-S	not available	Gram positive; <i>Clostridia</i>	PCE, TCE	Miller et al. (1997)
<i>Desulfotobacterium</i> sp. strain Y51	AB049340	Gram positive; <i>Clostridia</i>	PCE, TCE	Suyama et al. (2002)
<i>Desulfotobacterium frappieri</i> TCE1	X95972	Gram positive; <i>Clostridia</i>	PCE, TCE	Gerritse et al. (1999)
<i>Desulfotobacterium metallireducens</i>	AF297871	Gram positive; <i>Clostridia</i>	PCE, TCE	Finneran et al. (2003)
<i>Desulfuromonas chloroethenica</i>	U49748	<i>Proteobacteria</i> (subdivision)	PCE, TCE	Krumholz et al. (1996)
<i>Desulfuromonas chloroethenica</i> strain MS-1	not available	<i>Enterobacteriaceae</i>	PCE, TCE	Sharma and McCarty (1996)
<i>Desulfuromonas chloroethenica</i> strain TEA	not available	Low G+C Gram positive bacteria	PCE, TCE	Wild et al. (1996)
<i>Desulfuromonas michiganensis</i> strain BB1	AF357915	<i>Proteobacteria</i> (subdivision)	PCE, TCE	Sung et al. (2003)
<i>Dehalococcoides ethenogenes</i> strain 195	AF004928	<i>Chloroflexi</i> (green, non-sulfur bacteria)	PCE, TCE, <i>cis</i> -DCE, 1,1-DCE, 1,2-DCA, 1,2-dibromoethane, VC	Mayo-Gatell et al. (1997)
<i>Dehalococcoides</i> sp. strain FL2	P, AF357918	<i>Chloroflexi</i> (green, non-sulfur bacteria)	TCE, <i>cis</i> -DCE	Löffler et al. (2003)
<i>Dehalococcoides</i> sp. strain BAV1	P, AY165308 ³	<i>Chloroflexi</i> (green, non-sulfur bacteria)	<i>cis</i> -DCE, trans-DCE, 1,1-DCE, VC, vinyl bromide, 1,2-DCA	He et al. (2003)
Organisms Detected in Mixed Cultures				
<i>Dehalococcoides</i> sp. strain VS	V, AF388550	<i>Chloroflexi</i> (green, non-sulfur bacteria)	<i>cis</i> -DCE, VC	Cupples et al. (2003)
<i>Dehalococcoides</i> sp. KB-1/PCE	P, AY146780 ²	<i>Chloroflexi</i> (green, non-sulfur bacteria)	PCE	E. Edwards, unpublished data
<i>Dehalococcoides</i> sp. KB-1/VC	P, AY146779 ²	<i>Chloroflexi</i> (green, non-sulfur bacteria)	TCE, <i>cis</i> -DCE, VC	E. Edwards, unpublished data
<i>Dehalococcoides</i> sp. clone DHC-kb1C	C, AF388539	<i>Chloroflexi</i> (green, non-sulfur bacteria)	not reported	Hendrickson et al. (2002)
<i>Dehalococcoides</i> sp. Pinellas	P, AY146781 ⁴	<i>Chloroflexi</i> (green, non-sulfur bacteria)	not reported	E. Edwards, unpublished data
<i>Dehalococcoides</i> sp. strain CBDB1	P, AF230641	<i>Chloroflexi</i> (green, non-sulfur bacteria)	1,2,3-TCB, 1,2,4-TCB, 1,2,3,4-TeCB, 1,2,4,5-TeCB, PCDD, PCE, TCE	Adrian et al. (2000); Bunge et al. (2003)

(after Major et al., 2003 and He et al., 2003)

Notes

¹ GenBank accession number; letter indicates 16S rRNA sequence group as described by Hendrickson et al. (2002); C - Cornell, P - Pinellas, V - Victoria

² Microorganism is present in KB-1

³ Microorganism is present in the Bachman Road culture

⁴ Microorganism is present in the Pinellas culture

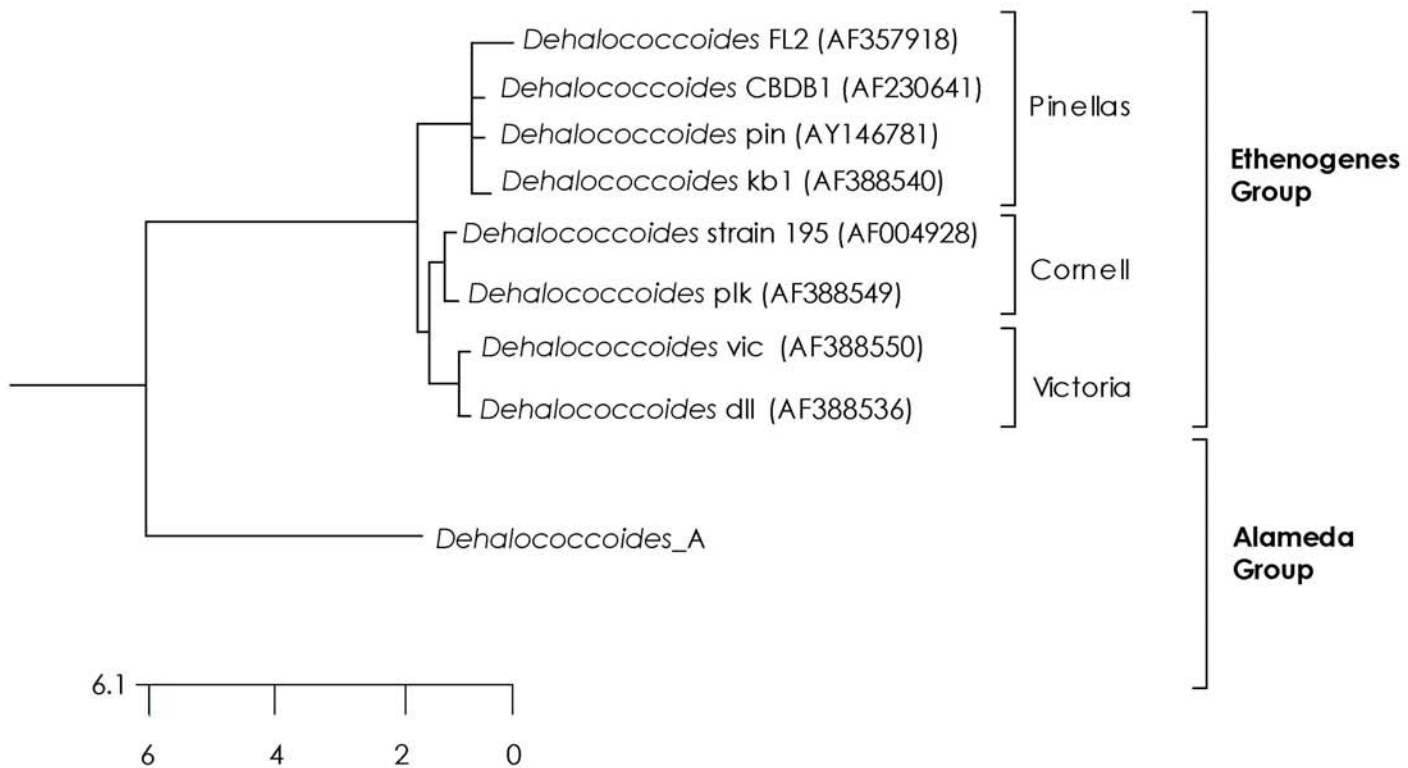
sites using a 16S rRNA molecular genetic method. While *Dehalococcoides* was present at all sites where dechlorination proceeded beyond *cis*-DCE to VC and ethene (21 of 24 sites), this microorganism was absent at all sites (3 of 24 sites) where dechlorination stalled, resulting in the accumulation of *cis*-DCE.

It is worth noting that even at sites where *Dehalococcoides* is present, dechlorination activity for chlorinated ethenes may not occur since some *Dehalococcoides* species are not able to utilize chlorinated ethenes as electron acceptors, suggesting that the occurrence of *Dehalococcoides* organisms with the requisite chlorinated ethene-degrading activity may be less widespread than suggested by Hendrickson *et al.* (2002). Finally, at sites where *Dehalococcoides* was not detected and dechlorination stalled at *cis*-DCE, the addition of mixed cultures containing *Dehalococcoides* has led to complete dechlorination to ethene and the establishment of this organism in the bioaugmented aquifer (*e.g.*, Ellis *et al.*, 2000; Cox *et al.*, 2002; Major *et al.*, 2002). These findings all suggest that *Dehalococcoides* with the requisite dechlorination activity is not ubiquitous in groundwater environments.

4.2 Phylogeny, Origin, and Characteristics of *Dehalococcoides* Organisms

The unique metabolic capabilities of *Dehalococcoides* warrant an examination of the evolutionary relationship of these microorganisms. The 16S rRNA sequences (a gene commonly used to identify evolutionary relationships between microorganisms) of *Dehalococcoides* and similar organisms have been isolated from ocean environments, contaminated aquifers, harbor sediment, anaerobic distillery sludge, freshwater sediments, and hot springs. Löffler *et al.* (2003) speculated that the detection of these sequences in open environments suggests that *Dehalococcoides* plays a role in biogeochemical cycling of halogenated organic compounds in marine ecosystems. Numerous *Dehalococcoides* 16S rRNA gene sequences have been analyzed to date, most of which were obtained from either environmental samples or mixed cultures. A search of GenBank (a comprehensive DNA sequence database) retrieved 50 individual *Dehalococcoides* 16S rRNA sequences. This list probably underestimates the diversity of the genus, as it does not include numerous other organisms likely affiliated with this genus but that were not defined as such when submitted to GenBank.

The *Dehalococcoides* belong to an isolated branch of the *Bacteria* phylogenetic tree containing organisms of the phylum *Chloroflexi* (green non-sulfur bacteria). In addition to *Dehalococcoides*, the phylum *Chloroflexi* contains several key genera, including *Chloroflexus* and *Thermomicrobium*, many of which are thermophilic and are often associated with hot springs (Madigan *et al.*, 2002). The *Dehalococcoides* 16S rRNA sequences are further categorized in sequence groups and sub-groups, which more precisely define the evolutionary relationships between organisms of this genus. The known *Dehalococcoides* sequences are divided into two groups, putatively defined as the Ethenogenes group and the Alameda group. The Ethenogenes group is further divided into three sub-groups, referred to as the Cornell (which includes the *Dehalococcoides ethenogenes* 195 isolate), Victoria, and Pinellas sequence subgroups (Hendrickson *et al.*, 2002). Figure 2 presents a dendrogram illustrating the relationships between the various *Dehalococcoides* groups and subgroups of some of the known *Dehalococcoides* organisms. The Alameda group is a recently identified *Dehalococcoides* cluster that falls distinctly outside of the Ethenogenes group (personal communication with S. Zinder, Cornell University).



*Dehalococcoides*_A = *Dehalococcoides*, Alameda group organisms

Figure reproduced by permission; Ed Hendrickson,
Corporate Remediation Group DuPont

Dendrogram of the Groups and
Subgroups of the Genus *Dehalococcoides*

Jan. 2005

Figure: 2



The widespread recognition of the significance of the isolation of the first member of the *Dehalococcoides* genus, “*Dehalococcoides ethenogenes* strain 195” (Maymo-Gatell *et al.*, 1999) has led to the near-generic use of the name “*Dehalococcoides ethenogenes*” to refer to any *Dehalococcoides* organism. The name “*Dehalococcoides ethenogenes*” or its abbreviation “DHE” refers to a particular species of *Dehalococcoides* isolated by researchers at Cornell University. While “*ethenogenes*” refers to the capability of this organism to dechlorinate to ethene (which was novel at the time of its discovery) this organism is an inefficient VC-dechlorinator in comparison to more recently described organisms such as *Dehalococcoides* BAV1 (He *et al.*, 2003a, He *et al.*, 2003b), *Dehalococcoides* VS (Cupples *et al.*, 2003) and *Dehalococcoides* KB-1/VC (Duhamel *et al.*, 2004).

Unfortunately, it is common practice for remediation professionals to use statements such as “tests for *Dehalococcoides ethenogenes* (DHE) indicated that this organism was present” when in fact the species is not necessarily “*ethenogenes*”. If interpreted literally, this statement implies that the species of *Dehalococcoides* isolated by researchers at Cornell University was present at the site, which is not likely the case at most sites. In most cases, unless referring to the organism isolated at Cornell, use of more general terms such as “*Dehalococcoides* organism”, “*Dehalococcoides* species”, or “member of the *Dehalococcoides* genus” is more appropriate.

Phylogenetic similarity based on 16S rRNA sequences, which lack sufficient resolution to provide species-level identification, is commonly interpreted to imply metabolic (phenotypic) similarity including specific electron acceptor requirements. While in certain cases this may be true, it is apparent that phylogenetic designations based on the 16S rRNA genes do not correlate well with phenotypic traits (Duhamel *et al.*, 2004). For example, although some members of the Pinellas subgroup use *cis*-DCE and VC as metabolic electron acceptors, another member of this sub-group (*Dehalococcoides* CBDB1) does not degrade these chloroethenes at all; instead this organism can degrade other recalcitrant contaminants including chlorobenzenes and polychlorinated dibenzodioxins (Bunge *et al.*, 2003).

Given the variability in dechlorinating activity, phenotypic assertions based on 16S rRNA phylogeny are subject to inconsistencies, although some general statements appear to apply to the metabolic capabilities of the *Dehalococcoides* sub-groups. For example, no members of the Cornell sub-group are known to derive energy from VC reduction (*e.g.*, *Dehalococcoides ethenogenes* 195), whereas some members of the Pinellas and Victoria (He *et al.*, 2003) sub-groups and Alameda group derive energy from this reaction (personal communication with E. Hendrickson, Dupont), ultimately increasing the biodegradation rate of this contaminant and limiting the tendency for accumulation of VC.

Additional information beyond the evolutionary phylogenetic information provided using only the 16S rRNA genes will ultimately be provided by analysis of other genes in the various *Dehalococcoides* subgroups. Due to its unusual traits, and the fact that it was the first *Dehalococcoides* isolated in pure culture, the entire genome of *Dehalococcoides ethenogenes* 195 (a member of the Cornell subgroup) was sequenced by the Institute for Genome Research (www.tigr.org). Sequencing has revealed that this organism possesses at least seventeen different genes that are potential dehalogenases (Villemur *et al.*, 2002). Entire genome analysis will ultimately provide a much greater understanding of the particular genes involved in dechlorination and the specific metabolic capabilities of individual *Dehalococcoides* organisms.

The *Dehalococcoides* strain BAV-1 has also recently been sequenced by the Joint Genome Institute (U.S. Department of Energy), and JGI has plans to sequence other strains as well.

Recent research on the genetics of dechlorination has shown that multiple, non-identical copies of reductive dehalogenase genes exist in different strains, with conserved regions that can allow development of gene probes to identify the genetic capabilities at a particular site (Holscher et al., 2004). However, the final step in anaerobic dechlorination, vinyl chloride dehalogenation to ethene, is catalyzed by a vinyl chloride reductase (vcr) enzyme, and there appear to be numerous vcr genes (Muller et al., 2004; Krajmalnik-Brown et al., 2004), complicating the potential for a simple test for this critical step in the anaerobic biodegradation of chlorinated solvents.

One fascinating aspect of *Dehalococcoides* organisms (and certain other dehalorespiring isolates, such as *D. restrictus*), is that electron acceptors for this group other than organohalogenes have not been identified to date. While there are natural sources of halogenated compounds, the highest environmental concentrations of these compounds are anthropogenic. One question that arises is, how did these organisms evolve and does their non-anthropogenic electron acceptor (if one exists) differ from the man-made compounds, or did these organisms evolve to degrade the lower concentrations of these compounds found naturally? Seshadi *et al.* (2005) suggest that *Dehalococcoides* may have recently evolved from a nitrogen-fixing autotroph.

Nonetheless, the apparently restricted metabolism of *Dehalococcoides* has potential advantages from the point of view of bioaugmentation. First, growth of these organisms is necessarily directly linked to dechlorination. Secondly, dechlorination can proceed in the presence of potentially competing electron acceptors such as sulfate, especially if an ample supply of hydrogen is present, although sulfate inhibition is observed at low hydrogen thresholds (Heimann *et al.*, 2004).

4.3 Conclusions Regarding the Microbiology of Dehalorespiration

Major *et al.* (2003) provides a short review of the current conclusions about the microbiology of reductive dechlorination drawn from the peer-reviewed literature. Several of the main conclusions are reiterated below:

- Many microorganisms have been isolated in pure culture that can reductively dechlorinate PCE and TCE as terminal electron acceptors during metabolism, and obtain energy from the process for cell growth;
- Dechlorinators live as part of an anaerobic microbial community, where fermenting, acetogenic, and other microorganisms contribute electron donor (hydrogen), organic carbon (*e.g.*, as acetate), and possibly other nutrients to the dechlorinating microorganisms (*e.g.*, cobalt-containing cofactors; Florencio *et al.*, 1994);
- All laboratory cultures that dechlorinate PCE or TCE beyond *cis*-DCE to ethene contain organisms in the genus *Dehalococcoides* (Maymo-Gatell *et al.*, 1997; Adamson and Parkin, 2000; Ellis *et al.*, 2000; Fennell *et al.*, 2001; Duhamel *et al.*, 2002; Richardson *et al.*, 2002; Cupples *et al.*, 2003; Dennis *et al.*, 2003; He *et al.*, 2003);

- Many *Dehalococcoides* 16S rRNA gene sequences have been analyzed to-date. Although they are similar, they are not identical. These sequences fall into four clusters, designated by Hendrickson *et al.* (2002) the Cornell (containing *Dehalococcoides ethenogenes* 195), Victoria, and Pinellas sub-groups;
- *Dehalococcoides ethenogenes* 195 obtains energy from all chloroethene dechlorination steps (PCE to TCE, TCE to DCE, DCE to VC) with the exception of the final step from VC to ethene (Maymo-Gatell *et al.*, 1999; Maymo-Gatell *et al.*, 2001), which occurs cometabolically, resulting in the accumulation of VC and slower conversion of VC to ethene;
- Some mixed cultures rapidly dechlorinate PCE or TCE to ethene with little accumulation of intermediates. These cultures demonstrate sustained dechlorination of VC to ethene, when supplied with only VC as electron acceptor (Duhamel *et al.*, 2002; He *et al.*, 2003a). Such cultures likely use VC as a growth substrate. Recently, through molecular monitoring of *Dehalococcoides* growth kinetics, it was demonstrated that *Dehalococcoides* VS obtains energy from VC dechlorination (Cupples *et al.*, 2003). Löffler *et al.* (2003) isolated a *Dehalococcoides* species (designated BAV1) that obtains energy from VC dechlorination to ethene. The *Dehalococcoides* 16S rRNA gene sequences from these VC dechlorinating cultures fall into either the Pinellas or Victoria sub-groups;
- Not all *Dehalococcoides* dechlorinate chloroethenes. Some, such as CBDB1, dechlorinate chlorobenzenes (Adrian *et al.*, 2000) and polychlorinated dibenzodioxins (Bunge *et al.*, 2003), while more distant relatives dechlorinate polychlorinated biphenyls (Wu *et al.*, 2002); and
- The presence of *Dehalococcoides* does not necessarily indicate that complete chloroethene reduction to ethene will occur. However, the converse appears to be true: if *Dehalococcoides* is absent, then dechlorination past *cis*-DCE and VC to ethene does not occur (Hendrickson *et al.*, 2002).

The above discussion indicates that:

1. *Dehalococcoides* occupies a unique environmental niche and therefore, will survive and compete with other dechlorinating bacteria; and
2. Identification of *Dehalococcoides* at a field site by the 16S rRNA gene sequence suggests the potential for dechlorinating activity, but is insufficient in itself because of the difficulty in associating phenotypic activity with strain-level identification (He *et al.*, 2003a; Duhamel *et al.*, 2004), suggesting that complementary evidence (*e.g.*, microcosms, appropriate field data) is required to conclusively assess the extent of dechlorinating activity.

4.4 Emerging Bioaugmentation Applications of Dehalorespiration

Anaerobic reductive dechlorination of chloroethenes (*e.g.*, PCE, TCE, DCE, VC), when linked to energy yielding respiratory metabolism (as opposed to cometabolism) is commonly referred to as

dehalorespiration. Dehalorespiration also encompasses the reductive metabolism of other chlorinated and brominated compounds. Dehalorespiration can also refer to reactions other than reductive dehalogenation including dihaloelimination (Smidt and de Vos, 2004) which is particularly relevant to the reduction of chlorinated ethanes including 1,1,2-trichloroethane, 1,1,2,2-tetrachloroethane (Lorah and Voytek, 2004).

Bioaugmentation is currently overwhelmingly focused on the dehalorespiration of chlorinated ethenes by *Dehalococcoides* organisms, although in the future anaerobic bioaugmentation with dehalorespiring bacteria will undoubtedly be applied to compounds other than the chloroethenes. For example, there appears to be significant potential for utilizing *Dehalococcoides* organisms for *in situ* biodegradation of chlorinated benzenes, ethanes, and propanes, polychlorinated biphenyls, dioxins, and brominated ethenes and ethanes (Table 2).

In particular, the dechlorination of some chloroethanes represents a promising target for bioaugmentation with *Dehalococcoides*. One common contaminant, 1,2-dichloroethane (1,2-DCA), has been produced in larger quantities than any other chlorinated hydrocarbon and is a suspected carcinogen (De Wildeman *et al.*, 2003). 1,2-DCA can be degraded either aerobically or anaerobically. Reports of aerobic degradation of 1,2-DCA in the literature include degradation by *Xanthobacter flavus* via hydrolytic dechlorination (Song *et al.*, 2004) and by *Pseudomonas* sp. strain DCA1, which was bioaugmented into a membrane aerated biofilm reactor (Hage *et al.*, 2004).

Anaerobic degradation of 1,2-DCA has been demonstrated by *Dehalococcoides ethenogenes* 195 and linked to growth, suggesting it is not simply a cometabolic process (Maymo-Gatell *et al.*, 1999). Furthermore, 1,2-DCA degradation has been demonstrated in other mixed anaerobic cultures (Duhamel *et al.*, 2002). The anaerobic degradation of 1,2-DCA typically proceeds by dihaloelimination to an unsaturated alkene (ethene in the case of 1,2-DCA; Lorah and Olsen, 1999). Recently, an organism unrelated to *Dehalococcoides* named *Desulfitobacterium dichloroeliminans* strain DCA1 was isolated in pure culture that derives energy from the dihaloelimination reaction of 1,2-DCA to ethene (De Wildeman *et al.*, 2003). Anaerobic degradation of 1,2-DCA under field conditions has also been observed (Nobre *et al.*, 2004). Given that biodegradation of 1,2-DCA is possible by aerobic and anaerobic means and that high concentrations of 1,2-DCA are present in groundwater in some chemical refining and manufacturing centers (*e.g.*, southwestern Louisiana) development and/or use of existing bioaugmentation cultures for remediation of this contaminant at field sites is likely.

Recently, reductive dechlorination of 1,1,1-TCA coupled to growth was documented for *Dehalobacter* sp. str. TCA1 (Sun *et al.*, 2002). Regenes has licensed the use of this strain. BCI has also reported isolation of a strain capable of TCA biodegradation. In this process, 1,1,1-TCA is reduced via 1,1-dichloroethane (1,1-DCA) to chloroethane (CA) (Figure 1b). Unfortunately, the dechlorination process appears to stop at CA (which is still considered a hazardous chlorinated compound) rather than further dechlorination to non-toxic ethane. Currently there is no evidence for the degradation of 1,1,1-TCA by *Dehalococcoides*, and this compound inhibits the dechlorination of chlorinated ethenes when present as a co-contaminant (Duhamel *et al.*, 2002). There are also no convincing reports of organisms or cultures that reductively dechlorinate CA to ethane, despite the general industry perception that this compound degrades easily.

There are several reports of complete degradation of 1,1,2,2-tetrachloroethane (1,1,2,2-TeCA) by mixed consortia (*e.g.* Chen, *et al.*, 1996; Lorah *et al.*, 1999). The degradation of 1,1,2,2-TeCA can involve both dihaloelimination and reductive dechlorination reactions. Dihaloeelimination produces *cis*-1,2- and *trans*-1,2-DCE isomers, which can be dechlorinated via VC to ethene. 1,1,2,2-TeCA can also dechlorinate via 1,1,2-TCA and 1,2-DCA to CA, where the reaction appears to stall as with 1,1,1-TCA.

The above examples briefly illustrate some of the untapped potential of using both *Dehalococcoides* and other dehalorespiring bacteria for bioaugmentation. As our understanding of dehalorespiration processes and microbiology grows, the number situations where these microorganisms can be used for bioremediation will also increase.

5. THE BUSINESS OF BIOAUGMENTATION

With improved understanding of the microbiology of chlorinated solvent biodegradation, and the completion of several highly monitored and successful field demonstrations of bioaugmentation, several microbial inoculants and vendors have emerged to support the growing practice of bioaugmentation to treat chlorinated ethenes. This section provides a review of the bioaugmentation cultures currently in field use (Section 5.1), and discusses: issues related to the performance of these cultures (Section 5.2); available pathogenicity data for bioaugmentation cultures in field use (Section 5.3); information related to quality assurance/quality control (QA/QC) practices for commercially-available bioaugmentation cultures (Section 5.4); survival of bioaugmented microorganisms (Section 5.5); and environmental factors influencing the transport of bioaugmented organisms in groundwater (Section 5.6).

5.1 Bioaugmentation Cultures Used in Field Demonstrations

To date, field-scale bioaugmentation projects for chlorinated solvent remediation have employed: i) defined cultures capable of aerobic cometabolic dechlorination of TCE and other chlorinated VOCs; ii) a specific denitrifying strain of *Pseudomonas* (a facultative aerobic organism) with the unusual and to date unique trait of also being able to transform CT to CO₂ via an iron-limitation induced siderophore; and iii) anaerobic dehalorespiring enrichment cultures for various chlorinated ethenes and ethanes (Table 3). Of these, the use of anaerobic dehalorespiring enrichment cultures is the fastest bioaugmentation deployment area, and has the widest potential for use at DoD sites, and therefore, this section focuses on these dehalorespiring cultures.

To obtain data on these bioaugmentation cultures, ESTCP forwarded a questionnaire to the primary commercial vendors of these cultures, and followed up with direct contacts. The questionnaire solicited information relating to several key areas, including: culture origin; culture production and QA/QC; and culture use at field sites. A copy of the questionnaire is provided in Appendix B. Responses to the questionnaire and follow-up questions are summarized in Table 4, and discussed below.

Based on the vendor survey, several enriched consortia containing *Dehalococcoides* have been used in field demonstrations described in the peer-reviewed scientific literature, including KB-1™, the Bachman Road culture (source of BAV-1 and BDI™), and the Pinellas culture. Several other cultures have been developed for which varying degrees of information are available. Information related to the enrichment, development, use and performance of these cultures is summarized below.

- KB-1™ is an enriched culture developed by Dr. Elizabeth Edwards at the University of Toronto. It is currently marketed by SiREM Laboratories (www.siremlab.com). According to SiREM, KB-1™ has now been injected into the subsurface at more than 23 sites in twelve states (and one site in Denmark). The successful use of KB-1™ has been documented by Major *et al.* (2002), Cox *et al.*, (2002), and Battelle (2004), among others.

Table 3: Bioaugmentation Cultures and Mixed Consortia Used for Treatment of Chlorinated Solvents in Groundwater

Bioaugmentation Cultures/ Consortia	Developer	Culture Source	Target Contaminants	Commercial Vendors	Primary Degradation Pathway	Key Microbial Species	Growth Conditions	Pathogenicity	Key References
Pure Cultures									
<i>Burkholderia cepacia</i> PR1 ₃₀₁	Camp, Dresser, & McKee and Colorado State University	Not specified	TCE	No vendor	Cometabolic oxidation	N/A	Aerobic basal salts medium (7.2 g/L sucrose, yeast extract) at pH=6.0, maintained at 30 C	USEPA has determined that <i>B. cepacia</i> has the potential to cause severe infection in sensitive populations (cystic fibrosis patients)	Bourquin et al. (1997)
<i>Burkholderia cepacia</i> ENV435	Envirogen	Adhesion-deficient strain developed by Envirogen	TCE	No vendor	Cometabolic oxidation	N/A	Aerobic basal salts medium (1.6% sucrose) at pH=7.0, maintained on alternating batches of sucrose or phenol	USEPA has determined that <i>B. cepacia</i> has the potential to cause severe infection in sensitive populations (cystic fibrosis patients)	Steffan et al. (1999)
<i>Methylosinus trichosporium</i> OB3b	Lawrence Livermore National Laboratory	Pure culture obtained from a national culture inventory	TCE	No vendor	Cometabolic oxidation	N/A	Higgin's salts minimal medium lacking added copper	Non-pathogenic	Duba et al. (1996); personal communication with R. Knapp
<i>Pseudomonas stutzeri</i> KC	Michigan State University	Not reported	Carbon Tetrachloride	No vendor	CT degradation to CO ₂ , formate, and an unidentified nonvolatile product without producing chloroform	N/A	Inoculum aerobically grown on site in filter-sterilized groundwater (T=20C, pH=8.2, 10 mg/L phosphate, 1.6 g/L acetate). Acetic acid (50%) was used to maintain pH at 8.0-8.2	Not known (high density of "contaminant" organisms present in the culture)	Dybas et al. (1997); Dybas et al. (1998)

Table 3: Bioaugmentation Cultures and Mixed Consortia Used for Treatment of Chlorinated Solvents in Groundwater (continued)

Bioaugmentation Cultures/ Consortia	Developer	Culture Source	Target Contaminants	Commercial Vendors	Primary Degradation Pathway	Key Microbial Species	Growth Conditions	Pathogenicity	Key References
Enriched Consortia									
KB-1	E. Edwards (University of Toronto) and GeoSyntec Consultants	TCE-contaminated aquifer, Ontario	chloroethenes	SiREM	Reductive dechlorination (dehalorespiration)	<i>Dehalococcoides</i> , <i>Acetobacterium</i> , <i>Geobacter</i> sp., <i>Methanospirillum</i> , <i>Methanosaeta</i>	Anaerobic defined mineral media with TCE/methanol at 23 C pH = 7.0	Negative test results ¹	Duhamel et al. (2002); Major et al. (2002);
Pinellas	Remediation Technologies Development Forum	TCE-contaminated aquifer, Pinellas, FL	chloroethenes	Terra Systems	Reductive dechlorination (dehalorespiration)	<i>Dehalococcoides ethenogenes</i> and others by T-RFLP	Chloride free minimal media containing yeast extract/ lactate/ TCE at 24 C pH= 7.0	Not reported	Ellis et al. (2000)
Bachman Road culture (BC2, Bio-Dechlor)	F. Löffler (Georgia Institute of Technology)	PCE-contaminated aquifer, Oscoda, MI	chloroethenes	Regensis, Bioaug-LLC	Reductive dechlorination (dehalorespiration)	<i>Dehalococcoides</i> sp., <i>Desulfuromonas michiganensis</i> strain BRS1, <i>Desulfuromonas chloroethenica</i>	Inoculum was grown in anoxic bicarbonate-buffered mineral salts medium with lactate as electron donor and PCE as acceptor	Not reported	Löffler et al. (2000); He et al. (2002); He et al. (2003); Lendvay et al. (2003)
Multiple Mixed Cultures	Bioremediation Consulting Inc.	VOC-contaminated sites	chloroethenes, chloroethanes	Bioremediation Consulting Inc.	Reductive dechlorination (dehalorespiration)	BCI's cultures contain <i>Dehalococcoides</i> sp.	Not known	Negative test results ²	personal communication with M. Findlay, BCI

Notes

¹ Results of pathogenicity testing for KB-1 are reported at www.siremlab.com/detailed_kb1.asp

² Results of pathogenicity testing for BCI cultures are reported at www.bcilabs.com/s.bioaug.html

Table 4: Summary of Quality Assurance/Quality Control Practices for the Production of Commercially-Available Bioaugmentation Cultures

Quality Assurance/ Quality Control Practice	Bioaug LLC	Bioremediation Consulting Inc. (BCI)	Regenesis	SiREM	Terra Systems, Inc.
Dhc Cell Density	NA	Not monitored	DNR	Determined monthly and prior to shipment using quantitative PCR methods (~108 cells/mL)	DNR
Pathogenicity	NA	Certified free of Salmonella, Shigella, Listeria, Staphylococcus aureus, Pseudomonas, E.coli , and Enterococci (no information on monitoring frequency)	DNR	Certified free of Salmonella sp., Listeria monocytogenes , Vibrio sp., Campylobacter sp., Clostridia sp., Bacillus anthracis , Pseudomonas aeruginosa , Yersinia sp., pathogenic yeast and mold (includes Candida albicans, Aspergillus fumigatus), fecal coliforms, and Enterococci (monitored semi-annually)	DNR
Community Composition	Culture contains Dhc-like microorganisms (monitoring frequency not specified)	Cultures are functionally characterized (e.g., whether they contain methanogens or sulfate reducers) and contain Dhc -like microorganisms; stability of community composition is monitored (monitoring frequency not specified)	DNR	Determined monthly and prior to shipment using DGGE methods	DNR
VOC Degradation Activity	NA	The activity of the culture compared to that of the original enrichment (monitoring frequency not specified)	DNR	Monitored weekly in the fermentors and prior to shipment of each batch	DNR
Culture Shipment	Shipped in stainless-steel vessels equipped with appropriate valves to allow injection of the inoculum	NA	DNR	Shipped in stainless-steel vessels that permit culture injection under anoxic conditions	DNR

Notes

NA - information not provided by vendor

DNR - vendor did not respond to questionnaire

DGGE - denaturing gradient gel electrophoresis

PCR - polymerase chain reaction

- The Pinellas culture is an anaerobic enrichment from a DOE facility in Pinellas, Florida. It was developed by Dr. Mark Harkness at General Electric Company's Corporate Research & Development center, and was used as the bioaugmentation culture for the field demonstration at Dover Air Force Base (AFB) in Delaware (Ellis *et al.*, 2000). Terra Systems (www.terrasystems.net) has licensed the Pinellas culture and developed it for commercial applications.
- Bio-Dechlor INOCULUM™ (BDI) is a chlorinated ethene detoxifying consortium that features multiple *Dehalococcoides* (DHC) strains including the ethene producing BAV1 strain (developed by Dr. Frank Löffler). It is currently marketed by Regenesis (www.regenesis.com). The successful use of the Bachman Road culture was reported by Lendvay *et al.* (2003). Regenesis reports that this culture has been used at 27 sites in 14 states (see Ritalahti *et al.* 2005; Sharma *et al.*, 2003).
- SDC-9 is a culture containing *Dehalococcoides* sp. that was enriched in the Knoxville, TN Technology Application Group laboratory of Shaw Environmental, Inc. (Shaw) (www.shawgrp.com). It is currently produced in quantities up to 4000 L per batch in the Shaw Lawrenceville, NJ facility (formerly Envirogen, Inc). The culture has been used for in situ treatment at more than 12 sites, and it was used successfully to inoculate an anaerobic fluid bed bioreactor treating mixed chlorinated solvents. Shaw also is currently developing several dehalorespiring microbial cultures by enriching them from contaminated sites and then re-applying them at those sites to accelerate remediation, although the enrichments may be used at other sites. Another culture, ENV-TCA20, has been enriched at the Shaw Lawrenceville, NJ laboratory for its ability to degrade chlorinated ethanes. This culture appears to contain a *Dehalobacter* strain similar to strain TCA1 (Sun *et al.*, 2002) based on 16s rRNA gene analysis, but it has not yet been applied in the field. (personal communication with R. Steffan, Shaw).
- BC2, a bioaugmentation culture marketed by BioAug LLC (www.bioaug.com), is an enrichment of the Bachman Road culture. Independent testing of the inoculum determined that it contains high densities (10^{10} cells/L of *Dehalococcoides* microorganisms). BioAug also provides blended aquifer conditioners based on site-specific geochemical conditions and CVOC concentrations. BioAug is under contract to perform bioaugmentation field demonstrations and has developed enriched cultures for site-specific treatment of sites with mixtures of CVOCs in addition to chlorinated ethenes (personal communication with R. Steele, BioAug LLC).
- Bioremediation Consulting Inc. (BCI; www.bcilabs.com) markets a number of bioaugmentation cultures acclimated to site-specific conditions that are not described in the peer-reviewed literature. BCI's chloroethene-degrading cultures contain "*Dehalococcoides ethenogenes*" microorganisms (personal communication with M. Findlay, BCI) although it may be that this generic nomenclature derives from each culture's production of ethene and is not intended to indicate a species-level likeness to *Dehalococcoides ethenogenes* 195. Recently, the culture has been tested by an independent lab, and the results

showed both high densities of *Dehalococcoides* as well as the presence of vinyl chloride reductase (using vcr probes). BCI cultures have been applied at six sites.

There is a limited understanding of the diversity of microorganisms in each of these cultures. For example, only two microorganisms, including a *Dehalococcoides* (*Dehalococcoides* BAV1) and *Desulforomonas michiganensis* strain BRS1, have been reported in the Bachman Road culture (Lendvay *et al.*, 2003). Community composition studies of KB-1™ indicate that two *Dehalococcoides*, in addition to *Acetobacteria*, *Sulfurospirillum*, *Hipaea*, and an uncultured soil bacterium PBS-111-32a are the dominant microbial species (Duhamel *et al.*, 2002), although methanogens are also present (personal communication with E. Edwards, University of Toronto). Similarly, BCI and Regenesis report that their cultures are mixed cultures, containing sulfate reducers and methanogens as well as *Dehalococcoides*.

Both KB-1™ and the Bachman Road culture *Dehalococcoides* fall within the Pinellas sequence sub-group. As shown in Table 2, at least two *Dehalococcoides* (KB-1/PCE and KB-1/VC) have been identified in KB-1™. KB-1/VC has been isolated in an enriched culture and has been demonstrated to grow on TCE, *cis*-DCE, and VC (Duhamel, *et al.*, 2004). An additional *Dehalococcoides* (KB-1C), a member of the Cornell sub-group, was detected in the original enrichment from which KB-1™ was derived (Hendrickson *et al.*, 2002), although this microorganism is not present in the enriched culture. *Dehalococcoides* BAV1 metabolically dechlorinates all DCE isomers and VC to ethene but does not appear to dechlorinate other chlorinated ethenes; however, this organism is also known to dechlorinate 1,2-DCA and vinyl bromide (He *et al.*, 2003a).

Isolated *Dehalococcoides* cultures, including *Dehalococcoides ethenogenes* 195, *Dehalococcoides* CBDB1, *Dehalococcoides* FL-2, and *Dehalococcoides* BAV-1, are very important for defining the metabolic function of these organisms in mixed cultures, although none of these organisms grow as well in pure culture as they do in mixed cultures. Obviously, these microorganisms participate in syntrophic relationships although little is known about the specific nature of the apparent dependency of *Dehalococcoides* on other organisms. The niche occupied by *Dehalococcoides* is similar to the one occupied by other hydrogen-utilizing anaerobes, such as sulfate-reducers and methanogenic bacteria, and these microorganisms are often co-enriched with *Dehalococcoides* in laboratory cultures. Various soluble electron donors have been used to enrich dechlorinating cultures, including methanol, ethanol, lactate, benzoate, and butyrate. Some enrichment cultures are maintained on hydrogen, but soluble electron donors are much simpler to feed to cultures and provide both energy and a carbon source for growth.

A number of other dechlorinating cultures have been enriched for use in laboratory studies and/or field demonstrations, although varying degrees of characterization data are available for these cultures. Mixed cultures derived from either field samples or sewage sludge are being widely employed in the academic community for bench-scale studies, including evaluations of novel electron donors such as tetrabutoxysilane (Yu and Semprini, 2002), meso-scale laboratory studies of enhanced bioremediation processes (Carr and Hughes, 1998; Isalou *et al.*, 1998), and mass transfer assessments (Yang and McCarty, 1998). Although the primary focus of these efforts is to increase the understanding of the composition and function of dechlorinating cultures it is also possible that commercial bioaugmentation products may eventually emerge from these research cultures.

Despite the existence of a number of commercial and research cultures, safe and effective application of bioaugmentation on a commercial/field scale requires vigilance beyond simply ensuring effective dechlorination. Large-scale culture production should maintain consistent microbial density, performance and composition, ensure the absence of pathogenic or opportunistic microorganisms, utilize delivery vessels and protocols that preclude exposure to oxygen and provide at least some understanding of culture performance under a wide range of site conditions. Specific approaches for ensuring that appropriate manufacturing and application standards are met are discussed in further detail in the following sections.

5.2 Factors Known to Affect Culture Performance

Several factors have the ability to influence the establishment and performance of bioaugmentation cultures introduced into groundwater environments. These include exposure to oxygen, competition for electron donor involving other electron acceptors, type and concentration of electron donor used, temperature and pH, concentration of the chlorinated solvents, and presence of other chlorinated solvents. These factors are briefly discussed below.

5.2.1 Oxygen Tolerance

Dehalococcoides are strictly anaerobic microorganisms (Maymo-Gatell *et al.*, 1997) and oxygen toxicity significantly impacts culture viability. He *et al.* (2003) reported that modification of laboratory protocols to minimize the exposure of the culture to oxygen was required to decrease the variability between replicate culture aliquots, suggesting that even minimal oxygen exposure may be problematic. The presence of oxygen in the headspace of microcosm bottles (0.7% v/v, corresponding to dissolved oxygen concentration of approximately 0.3 mg/L) significantly decreased the rate of TCE degradation (Seepersad, 2001). Several manufacturers have developed delivery approaches and injection protocols that prevent/limit exposure to oxygen during delivery and during transfer of the products into the sub-surface. Regenesi delivers Bio-Dechlor Inoculum™ (BDI) in airtight, 5-gallon retrofitted buckets that are then sparged on-site with nitrogen to remove any residual chlorinated solvents prior to injection. After sparging, the culture is then mixed in a 1:100 ratio with chemically reduced site groundwater to increase the injection volume and allow for better distribution of the culture within the subsurface. Delivery and injection of KB-1™ is accomplished using specialized stainless steel shipping canisters designed to maintain anaerobic conditions, and the culture is injected into the subsurface by pressurizing the vessel using argon gas once anaerobic and reducing redox conditions have been achieved (Major *et al.*, 2002; personal communication with P. Dennis, SiREM). A similar system is used by BCI. Shaw supplies SDC-9 in 20-L stainless steel soda kegs that allow simple pressurized delivery of the culture. Shaw, however, first concentrates their SDC-9 culture approximately 10-fold by using an anaerobic membrane filtration device. This process removes 90% of fermentation byproducts and any remaining substrate (e.g., PCE etc.), reduces shipping volumes and costs, and allows large volumes of culture to be economically shipped overnight on ice to ensure activity.

5.2.2 Geochemical Conditions

Apart from oxygen sensitivity, geochemical variability can impact the performance of bioaugmentation cultures in the field. The establishment of the appropriate redox conditions is essential for effective bioaugmentation. Redox is influenced by the presence of alternate electron acceptors including nitrate, manganese/iron oxides and sulfate. In general, reductive

dechlorination is favored under sulfate reducing or methanogenic conditions (AFCEE, 2004), suggesting that oxygen, nitrate and manganese/iron oxides will be reduced prior to bioaugmentation. Successful bioaugmentation and TCE dechlorination to ethene has been reported at an anaerobic site with elevated concentrations of nitrate (25 mg/L) and perchlorate (15 mg/L), all of which were removed due to electron addition ([AFCEE, 2004](#)).

Apparent sulfate inhibition has been observed in several cases, but the issue has been a confusing one. For example, complete dechlorination of TCE to ethene was not observed in bioaugmented microcosms (Pinellas culture) containing sulfate at 3,000 to 6,000 mg/L, despite active sulfate reduction, re-bioaugmentation with the Pinellas culture, and application of multiple electron donors (ESTCP project CU-9914: www.estcp.org). Heimann *et al.* (2004) observed no sulfate inhibition when electron donor was present in excess, although 240 mg/L of sulfate inhibited dechlorination when electron donor concentrations were limiting. In contrast, groundwater at the site of origin for KB-1TM contained more than 1,000 mg/L of sulfate, and SDC-9 was successfully applied in the presence of high sulfate concentrations (> 1,000 mg/L) at Treasure Island NAS.

Recent work has cleared up some of this confusion. However, by showing that sulfide (naturally present or formed during sulfate reduction) is actually responsible for the toxic effects. This sulfide toxicity can be alleviated by precipitating the sulfide into unavailable mineral forms, for example by natural or added iron (Hoelen and Reinhard, 2004; Jeong and Hayes, 2003; <http://www.estcp.org/projects/cleanup/2002260.cfm>).

The roles of iron and manganese reduction on bioaugmentation performance have also not been sufficiently investigated, but the presence of ferric iron at some sites is thought to inhibit dechlorination (Koeingsberg *et al.*, 2002). As with most microbial processes, groundwater pH can affect dehalorespiration. KB-1TM, for example, exhibits no dechlorination below pH 5 and above pH 10, and the optimal pH for dechlorination is between 6.0 and 8.3 (Rowlands, 2004).

Finally, low groundwater temperatures in northern climates will undoubtedly slow dechlorination rates, although KB-1TM was active in groundwater at its site of origin where groundwater temperatures reach as low as 4°C in the winter. KB-1TM has been demonstrated to be capable of complete dechlorination to ethene in microcosms at temperatures as low as 10°C, while its maximum dechlorination rate occurs between 20-30°C (personal communication with A. Fris, Technical University of Denmark). Furthermore bioaugmentation has been performed at a site in Alaska where groundwater temperatures range from 4 to 8°C, resulting in slow dechlorination to ethene (personal communication with P. Dennis, SiREM).

5.2.3 Electron Donor Selection

Given the diversity and the metabolic flexibility of fermentative organisms contained within mixed consortia, most bioaugmentation cultures can utilize a wide variety of electron donors in fermentative processes. By far the most commonly used donors to date have been HRCTM, molasses, vegetable oil, and lactate solutions. Bioaugmentation has apparently been performed successfully with all of these common donors.

Although the specific fermentation products produced by electron donors depend on the particular electron donor, molecular hydrogen, acetate and propionate are common (Fennell *et*

al., 1997). Propionate can be further fermented by some organisms to produce acetate, carbon dioxide, and molecular hydrogen (Liu *et al.*, 1999). Acetate can be readily utilized for growth by sulfate-reducers and methanogens, as well as some dechlorinators. For example, *Desulfuromonas chloroethenica* uses acetate directly as an electron donor for the dechlorination of PCE and TCE to *cis*-DCE (Krumholz *et al.*, 1997). Duhamel *et al.* (2002) reported that KB-1TM readily utilized methanol, ethanol, hydrogen, lactate, and propionate to support dechlorination, although acetate, which is not a fermentable hydrogen source, did not support dechlorination.

Dehalococcoides BAV1, which is capable of utilizing VC and DCE isomers as metabolic electron acceptors, readily utilized hydrogen but not formate, acetate, lactate, pyruvate, propionate, glucose, ethanol, or yeast extract as a electron donor (He *et al.*, 2003). The *Dehalococcoides* depend on hydrogen as the sole electron donor for dechlorination (Löffler *et al.*, 2003), emphasizing the significant role of the non-*Dehalococcoides* microorganisms present in mixed consortia in supporting dechlorinating activity through the production of hydrogen. Although hydrogen is readily used by methanogenic bacteria, several studies suggest that *Dehalococcoides* competitively utilize hydrogen at concentrations below those supporting methanogenesis (Smatlak *et al.*, 1996; Yang and McCarty, 1998; Löffler *et al.*, 1999).

5.2.4 VOC Concentration

Although the prevailing paradigm that bioremediation processes were ineffective in high concentration DNAPL source areas has historically restricted the application of the technology to plume containment or treatment (Pankow and Cherry, 1996), recent data demonstrate that dechlorinating microorganisms are active over a wide range of chloroethene concentrations. Duhamel *et al.* (2002) reported that KB-1TM promoted dechlorination of PCE, TCE, *cis*-DCE and VC at initial concentrations of 132, 197, 77, and 87 mg/L, respectively, in microcosm studies. More recent data suggest that the culture maintained dechlorinating activity at concentrations of PCE and TCE as high as 180 and 400 mg/L, respectively (personal communication with E. Edwards, University of Toronto). In a column inoculated with TM-1, a dechlorinating culture derived from anaerobic digester sludge, PCE dechlorination was sustained at influent concentrations as high as 99 mg/L (Isalou *et al.*, 1998).

Similar results were reported by Yang and McCarty (2000), who observed PCE dechlorination in the presence of *cis*-DCE and ethene at concentrations of 0.66 and 1.05 mM (64 and 29 mg/L, respectively). The presence of such high concentrations of PCE, *cis*-DCE and ethene can be inhibitory to methanogenesis (Yang and McCarty, 2000), improving electron donor availability for dehalorespiration. The occurrence of dechlorinating activity, even at very high chlorinated solvent concentrations, suggests that bioremediation processes may be utilized as part of a DNAPL source remediation strategy. The use of bioaugmentation for DNAPL source zone treatment is an emerging technology application that is further discussed in Section 6.2.

5.2.5 Inhibition by Selected VOCs

While chloroethenes are inhibitory only at extremely high aqueous concentrations, several other VOCs have been shown, or are suspected, to exert inhibitory effects on some cultures at much lower concentrations. Both chloroform and 1,1,1-TCA slowed rates of VC dechlorination to ethene by KB-1TM, with complete inhibition at concentrations of 450 µg/L (3.8 µM) and 700 µg/L (5.2 µM), respectively (Duhamel *et al.* 2002). Comparable results are reported for other

chloroethenes, including inhibition of *cis*-DCE dechlorination at 190 µg/L (1.6 µM) chloroform (Maymo-Gatell *et al.*, 2001), and of PCE dechlorination at 1,000 µg/L (8,400 µM) chloroform (Maymo-Gatell *et al.*, 2001, after Carney, 1995). In a microcosm study performed to compare SDC-9 and the BCI culture, however, neither culture appeared to be inhibited in groundwater containing relatively high concentrations of CF (27 µM), 1,1,1 TCA (91 µM); TCE (300 µM); *cis*1,2-DCE (720 µM), and carbon tetrachloride (15 µM) (R. Steffan, personal communication).

Inhibition of some dechlorinating cultures by a common co-contaminant such as 1,1,1-TCA suggests that bioaugmentation cultures must be selected carefully, and that some cultures may be inappropriate for treating some mixed-waste disposal sites. It should be recognized, that organisms capable of 1,1,1-TCA biodegradation have been isolated (Sun *et al.* 2002) and may be commercially available as well. BCI has a culture capable of dechlorinating both TCA and TCE (S. Fogel, personal communication). Likewise, Shaw's SDC-9 has been shown to degrade both TCA and PCE, and their ENV-TCA20 culture can degrade TCA and DCA (R. Steffan, personal communication). Reductive dechlorination of 1,1,1-TCA to less chlorinated ethanes (1,1-DCA and chloroethane) can be stimulated in some sites via addition of electron donors alone, suggesting that it may be possible to reduce concentrations of 1,1,1-TCA in certain cases simply by biostimulation. In cases where the appropriate organisms are absent, bioaugmenting with a 1,1,1-TCA dechlorinator prior to bioaugmentation for chloroethene degradation could be performed.

5.3 Pathogenicity

The responsible use of bioaugmentation must consider the potential adverse impacts related to culture pathogenicity. Pathogenicity may be associated with the dechlorinating microorganisms themselves, or with other microorganisms present in the culture. Although enrichment from soil and groundwater, as compared to sewage sludge, decreases the probability of isolating a pathogenic organism, it does not eliminate this prospect (Govan *et al.*, 2000).

Of the microorganisms listed in Table 3, only *Burkholderia cepacia* is known to be of particular concern. *B. cepacia* is an opportunistic human pathogen implicated in nosocomial infections, particularly in patients with cystic fibrosis and other immuno-compromised individuals (Av-Gay, 1999). There is little distinction between the potential pathogenicity of the clinical and environmental isolates of this species, and the characteristic multiple chromosomes and insertional sequences present an unusual degree of adaptability and the potential for genetic exchange. In addition, this organism has a multi-drug resistant phenotype (Vidaver *et al.*, 1999; Govan *et al.*, 2000). Accordingly, USEPA has recently implemented comprehensive restrictions on the use of *B. cepacia* for purposes other than "research and development in the degradation of chemicals via injection into subsurface groundwater" (68 FR 35315-35320, June 13, 2003).

The other aerobic and anaerobic dechlorinating organisms (including *P. stutzeri*, *M. trichospina*, and *Dehalococcoides*) and other organisms commonly associated with bioaugmentation cultures (*e.g.*, methanogens, acetogens) are considered non-pathogenic. There is always a concern that other unknown or unidentified organisms exist in enrichment cultures, and the potential presence of human or animal pathogens cannot be completely ruled out. However, the major concern at this point is not the potential pathogenicity of *Dehalococcoides* or other strains present in the

original consortia used, but the potential for introducing pathogens during routine culturing and large-scale growth of cultures for bioaugmentation of field sites.

Table 4 summarizes the pathogenicity information that was provided by the bioaugmentation culture vendors through the questionnaire. BCI, Regenesis, Shaw, Terra Systems, and SiREM provided information on pathogen testing. In a strict sense, it is impossible to certify any mixed culture as “pathogen-free”, since there may be unknown pathogens. All cultures were certified to be free of common pathogens, though the frequency of testing and the extent of pathogen testing vary somewhat between companies (BCI, 2004; SiREM, 2003; R. Steffan, Shaw, personal communication; S. Koenigsberg, personal communication). Regulatory guidance on appropriate test protocols in this area would be welcome.

All of these companies report that their cultures are in fact consortia containing other microorganisms in addition to *Dehalococcoides*. In all cases, the cultures contain sulfate reducers and methanogens that are apparently common in anaerobic environments. SiREM and Shaw indicated that they perform denaturing gradient gel electrophoresis (DGGE) testing to identify the predominant organisms in the consortium, and to determine shifts in the microbial community that may indicate the presence of contaminant microorganisms inadvertently introduced during production.

5.4 Culture Quality Assurance/Quality Control

The potential for adverse impacts to groundwater quality during bioaugmentation caused by the inadvertent inclusion or introduction of pathogenic or opportunistic microorganisms represents a significant potential concern. Equally important is the potential for adverse impacts to the dechlorinating activity of a bioaugmentation culture (e.g., contamination of, or changes to, the microbial community composition). The implementation of QA/QC protocols to prevent these impacts will encourage the effective application of bioaugmentation and, in the longer term, increase the acceptance of bioaugmentation as a credible remediation technology.

At a minimum, protocols for the production and shipment of bioaugmentation cultures to field sites for application should ensure that:

- The degradative activity and composition of each batch of the culture is comparable with that of the original culture for which reliable field performance and characterization data are available;
- The degradative microorganisms in the culture are not displaced or reduced in number by pathogenic or other opportunistic microorganisms during production, shipment or introduction to the subject site;
- The composition and density of the culture is uniform between successive production batches; and
- The viability and activity of the culture at the time of injection are at the highest possible levels.

As shown in Table 4, the primary vendors of bioaugmentation consortia have developed slightly varying QA/QC processes to ensure product quality. In response to the survey and follow-up

questioning, Shaw, BCI, Regenesis, Terra Systems, BioAug LLC, and SiREM provided QA/QC protocol information. The survey results are summarized below:

- Shaw has the ability to produce dechlorinating cultures in volumes up to 4000 L, and uses 20-L, 750-L or 4000-L commercial-grade stainless steel, computer controlled fermentors for culture production. In addition to standard fermentor controls (e.g., temperature, pH, redox), fermentation progress is monitored by using qPCR to measure DHC concentrations, and bottle assays are used to monitor culture activity. Cultures are typically grown to $>10^{10}$ /L. After large-scale fermentation (>40 L) the cells are concentrated ~ 10 -fold under strict anaerobic conditions by using a sterilizable stainless steel and ceramic membrane concentrator. The concentrated culture is then transferred anaerobically and aseptically to sterilized stainless steel 20-L shipping containers that are placed on ice in coolers and shipped overnight to the target site. The cultures are injected in the field by the use of quick connect fittings and pressurizing the containers with nitrogen or argon. Culture integrity is verified frequently by performing DGGE analysis.
- BCI maintains several cultures isolated from various VOC-impacted field sites, which are primarily characterized in terms of metabolic activity (e.g., methanogenic, sulfate-reducing) and dechlorination rate and are certified “pathogen-free”. Cultures differ in their adaptations to various environmental factors such as salinity and sulfate concentrations. For site-specific applications, the cultures are routinely acclimated to site groundwater for several weeks to ensure the dechlorinators will be as active and adapted as possible to the site conditions. Further, testing is performed during culture production to ensure that the dechlorination rate of the production culture is comparable to that of the original enrichment. BCI cultures are shipped overnight in anaerobic 20-liter stainless steel pressure vessels or in 1-liter containers that facilitate transfer of the cultures to groundwater under anoxic conditions.
- SiREM has a detailed written program of standard operating procedures for the production of KB-1TM (personal communication with S. Dworatzek, SiREM). During culture production, quality control measures include media sterility checks, pathogen testing, monitoring of VOC concentrations to ensure ongoing degradation, monitoring of *Dehalococcoides* titer by quantitative PCR, and analysis using denaturing gradient gel electrophoresis (DGGE) to monitor the stability of the microbial community structure and to rule out the loss of key organisms or the introduction of pathogens. During culture growth and immediately prior to shipment, the TCE degradation activity of each batch of culture is confirmed in microcosms. Upon achieving the specified culture performance (i.e., consistent dechlorinating activity and a minimum *Dehalococcoides* cell density of 10^9 cells/L), KB-1TM is shipped overnight in sterilized anaerobic stainless steel pressure vessels that facilitate transfer of the culture to groundwater under anoxic conditions.
- Regenesis, produces its Bio-Dechlor INOCULUMTM (BDI) at Applied Power Concepts (APC, Anaheim, CA). The culture is grown in large reactors that scale

from 5 gallons to 10 gallons to 100 gallons and ultimately to 300 gallons. The culture is grown in mineral salts medium supplemented with a proprietary mix of simple natural organics additives. Additionally the culture is grown on sodium lactate and TCE which is replenished as needed. BDI contains multiple *DHC* species including the BAV1 strain that is responsible for efficient ethene production and detoxification. Other *DHC* organisms present in BDI were derived from contaminated site materials related to a long history of bioremediation and bench scale treatment studies conducted by APC for Regenesis. Typically, the BDI consortium contains 10^8 *DHC* cells/mL (10^{11} cells/L) and 10^6 cells/mL of the BAV1 strain (10^9 cells/L). Each reactor in which the BDI culture is grown is regularly tested for pathogens according to industry standards to ensure that all cultures are certified as pathogen-free for those pathogens tested. The pathogens BDI is tested for include a variety of coliforms, molds and yeasts, salmonella, and staphylococcus organisms. Claims that one culture is better than another based on this kind of testing are thin as additional tests are easy to add should a “competition” arise on this basis. Perhaps a regulatory authority can set standards for all to meet which would be welcome. RT-PCR tests are also regularly run on samples of the culture from each reactor to ensure high cell counts in every batch of BDI that is distributed. In order to ensure the survivability of the organisms during shipment to field sites, a complete QA/QC procedure was developed that verifies the viability and dechlorinating activity of the organisms at the time of injection. Samples are collected just before injection and analyzed for dechlorination activity in the laboratory.

- BioAug LLC has written standard operating procedures for inoculum production and in-field application of the BC2 inoculum. QA/QC testing is performed on an ongoing basis during inoculum production and before shipment of product to the field, including pathogen testing, monitoring of chlorinated ethenes and breakdown products, and monitoring of *Dehalococcoides* and the presence of other micro-organism species by PCR analysis. Inoculum production is performed in a series of stainless steel reactors ranging in volume from 5 to 100 gallons. Typically, the BC2 consortium contains a minimum cell count of 10^{10} *DHC* cells/L. BioAug also develops site-specific aquifer conditioner blends and inocula based on the geochemistry of a site and the mixture of CVOCs that are present at sites.

6. BIOAUGMENTATION IN THE FIELD

The scientific data characterizing dechlorinating microorganisms provides important insight into the conditions under which bioaugmentation may be an effective component of a site remediation strategy. However, the emerging nature of this technology suggests that the substantial benefit may be obtained through careful examination of the approaches employed in well-controlled field demonstrations.

The following sections include: a discussion of how the presence of an indigenous *Dehalococcoides* impacts the implementation of bioaugmentation; an overview of assessment methods to determine the site-specific need for bioaugmentation; brief discussions of the survival and transport of microorganisms added to the subsurface; summaries of field demonstrations of bioaugmentation; and an overview of the use of bioaugmentation for the remediation of DNAPL source zones. Detailed descriptions of several anaerobic and aerobic bioaugmentation field demonstrations are provided in Appendix C.

6.1 Deciding Whether Bioaugmentation Is Needed

The decision to bioaugment is a function of several factors, including economic, political, and technical considerations. From a technical perspective, it will be essential to bioaugment only at the relatively small number of sites that do not have an indigenous *Dehalococcoides* population. However, from an economic and political perspective, it may be appropriate to bioaugment at a much larger number of sites. It is critical, however, to realize that bioaugmentation is not a “magic bullet” that alone will solve any problematic site. Several geochemical factors can cause poor performance at sites with competent microbial populations. Some have therefore advocated a “diagnostic” approach to problem sites, in which microbial competence is only one of the potential limitations addressed (Koenigsberg et al., 2003).

There is considerable debate over whether bioaugmentation is beneficial. Some believe that at the vast majority of sites, the desired activities will occur, and it is simply a matter of “more time and more electrons” (e.g., Suthersan et al., 2002; Koenigsberg et al. 2003). The consensus opinion of most practitioners is that bioaugmentation is necessary to achieve complete dechlorination at some sites, but that these sites represent a relatively small fraction of the total number of chlorinated solvent sites. Most also agree that bioaugmentation will make economic sense at a larger fraction of the total number of sites, because the cost for adding organisms will be less than the cost of the increased time and electrons (Major et al., 2005). No definitive guidance on this decision-making process is possible at this time, but the key factors impacting the decision are discussed below.

Although costs for the culture solutions needed for bioaugmentation are decreasing, the inoculum itself can still be a significant expense at relatively large sites. A key economic consideration affecting the decision to bioaugment at any site is the need for active recirculation of the introduced culture. Most field-scale demonstrations of bioaugmentation have used a recirculation approach, which requires active pumping and extraction, with resulting labor and equipment costs. However, the recirculation approach has been used primarily to ensure rapid and thorough distribution for the purpose of demonstrating the technology’s efficacy in a short

time period. The vendors contacted all indicated that they had successfully used a much less costly passive injection approach for commercial applications, and believed it would be effective under most site conditions. However, rigorous demonstrations of the effectiveness of passive injection have not yet been performed.

The recent guidance on Enhanced Anaerobic Bioremediation (AFCEE, 2004: <http://www.afcee.brooks.af.mil/products/techtrans/Bioremediation/downloads/PrinciplesandPractices.pdf>) recommends that project managers explicitly evaluate the costs and benefits of bioaugmentation when designing and initiating a bioremediation project. Although bioaugmentation does increase the initial costs, it is critical to evaluate the benefits as well, on a life-cycle cost basis. The benefits can include reduced electron donor costs, reduce operations and monitoring time (and costs), and increased regulatory acceptance as performance is more rapidly demonstrated. The total cost for bioaugmentation has been estimated at 1-3% of the total treatment cost, and it may well pay for itself if it reduces the time until complete dechlorination is achieved by only a couple months (Major et al., 2005).

The most obvious use of bioaugmentation is at sites that completely lack the requisite *Dehalococcoides* microorganisms and/or where only partial dechlorination of PCE and TCE occurs. Clearly, there are sites where *Dehalococcoides* capable of complete dechlorination cannot be recovered, and complete dechlorination is not observed over even extended periods of donor addition (e.g., Hendrickson et al., 2002). At these sites bioaugmentation may be used to ensure that the necessary microorganisms to achieve complete dechlorination to ethene are present or to supplement the activity of the existing dechlorinating population.

However, even at sites where competent *Dehalococcoides* are present, bioaugmentation may be worth doing, to decrease the lag time prior to the onset of dechlorination. This is particularly true for sites desiring rapid remediation due to impending property transaction. For example, complete dechlorination to ethene following biostimulation with lactate was demonstrated at the Bachman Road site, where an indigenous *Dehalococcoides* population was present (Lendvay *et al.*, 2003). In a biostimulation plot, complete conversion to ethene occurred after twelve weeks of electron donor addition; bioaugmentation of an identical treatment plot shortened the lag time to six weeks, a benefit that may be significant when travel times to compliance points are insufficient, or where there are stringent regulatory or commercial deadlines. Further, bioaugmentation at these sites may be used to provide *Dehalococcoides* microorganisms with the metabolic capacity to utilize both *cis*-DCE and VC as electron acceptors, which may be absent in the indigenous *Dehalococcoides* population.

Bioaugmentation may also be appropriate at sites where the indigenous *Dehalococcoides* microorganisms are present but are non-uniformly distributed. Fennell *et al.* (2001) reported that the activity of an indigenous *Dehalococcoides* was non-uniformly distributed in a chlorinated ethene-contaminated aquifer at Cape Canaveral Air Force Station, Florida, reporting a direct correlation between the presence of *Dehalococcoides* and the ability to achieve complete dechlorination of *cis*-DCE and VC to ethene. These findings demonstrate that the detection of *Dehalococcoides* in a single location should not be used to infer the microorganisms are present throughout the aquifer.

The choice of electron donor can also affect the decision about whether or not to bioaugment. Using high-strength soluble donors (such as lactic acid or molasses) that have to be added at frequent intervals may make bioaugmentation more attractive, because it is relatively expensive to operate the system for even a few months without achieving complete dechlorination. On the other hand, bioaugmentation may be less attractive when using long-lasting, less soluble donors (such as chitin, HRCTM or vegetable oil), because the time and additional O&M needed to achieve complete dechlorination may represent a relatively small incremental cost.

Finally, the regulatory environment is a factor worth considering in the decision-making process. If bioaugmentation reduces the time and uncertainty involved, it may well reduce the monitoring and negotiating costs. On the other hand, some regulators are wary of adding organisms, particularly a mixed culture in which not all of the organisms are fully characterized, and in such cases bioaugmentation can increase the design and permitting time and costs.

6.2 Deciding When to Bioaugment

Regardless of the site-specific extent of dechlorinating activity, bioaugmentation may be employed at several points during the implementation of an enhanced bioremediation strategy. Currently, the most common application is the use of bioaugmentation as a contingency in the event that the indigenous microbial community does not express sufficient dechlorinating activity (*e.g.*, dechlorination stalls at cis-DCE) following electron donor addition.

However, others favor a “prophylactic” approach, in which organisms are added at the start of electron donor addition if there is any reason to suspect that complete dechlorination may not occur (*e.g.*, based on a combination of treatability and/or field evidence). Given current practices, the cost of bioaugmentation is low relative to the cost of electron donor addition, and the prophylactic approach may be preferable since it will improve the likelihood of promoting complete dechlorination and shorten the lag time prior to the onset of ethene formation. Further, from a technical perspective, bioaugmentation may well be more effective if done early, rather than allowing competing organisms to become established over several months of donor additions.

A typical bioaugmentation practice at industrial sites is to bioaugment immediately following electron donor addition to minimize the establishment of competitive, non-dechlorinating microorganisms that may deplete essential nutrients. As well, at field sites where groundwater is aerobic, an initial period of electron donor addition is typically used to deplete dissolved oxygen, which is toxic to *Dehalococcoides*, and develop reducing conditions prior to bioaugmentation. Once anoxic conditions are achieved, bioaugmentation may be performed as soon as sufficient electron donor is available in the subsurface to deplete other electron acceptors (*e.g.*, nitrate, iron or manganese oxides, and sulfate) and support dechlorinating activity.

6.3 Methods to Determine the Need for Bioaugmentation

Currently, there are three methods to assess the need for bioaugmentation including direct detection of *Dehalococcoides* using molecular analyses, microcosm testing, and interpretation of field geochemical evidence (*e.g.*, AFCEE, 2004). While these methods can be used

independently, combining assessment methods will lead to a stronger conclusion. The basis, benefits and limitations of each assessment method are discussed in the following sections.

6.3.1 Direct Detection

The unique growth requirements of *Dehalococcoides* and their syntrophic association with other anaerobic microorganisms preclude using conventional microbial detection techniques such as plate counts for the detection of these organisms. Instead, molecular biological techniques utilizing the polymerase chain reaction (PCR) are commonly employed to determine the presence or absence and to quantify the number of *Dehalococcoides* microorganisms in soil or groundwater via the 16S rRNA gene. Until recently standard electrophoresis-based PCR was used for tracking *Dehalococcoides*. The drawback of standard PCR is the results are not fully quantitative, despite there being a general correlation between the intensity of electrophoresis bands and the number of gene copies extracted from the sample (Hendrickson *et al.*, 2002). Quantitative PCR methods now commercially-available are fully quantitative and may be used to accurately determine the number of *Dehalococcoides* 16 S rRNA gene copies in a sample (Smits *et al.*, 2004).

Detection of *Dehalococcoides* by PCR methods may also provide information regarding the potential to achieve complete dechlorination at a site. As indicated previously, there are differences in the ability of different strains of *Dehalococcoides* to halo-respire chlorinated ethenes, and therefore strain identification may be useful. For example, studies have shown that VC production (*i.e.*, incomplete dechlorination) is associated with a Cornell sequence subgroup *Dehalococcoides* (Hendrickson *et al.*, 2002; Maymo-Gatell *et al.*, 1997). The presence of Cornell subgroup organisms can be confirmed through DNA sequencing of PCR products. Furthermore, the functional genes involved in vinyl chloride dechlorination (vinyl chloride reductase) have recently been to be discovered and sequenced (Seshadri *et al.*, 2005; Müller *et al.*, 2004; He *et al.*, 2003b). Sequencing has allowed the development of PCR-based tests for these important functional genes, which are directly linked to positive metabolic capabilities, and are also commercially available.

A complicating factor in the use of PCR methods is the interpretation of results below the detection limit. Negative detection of *Dehalococcoides* may result from the detection limit of the assay, although typical methods detect as few as 100 gene copies per Liter; however, a particular sample might not contain *Dehalococcoides* DNA, even at sites that contain this organism at other locations, due to sampling bias since the majority of biomass in groundwater is attached to soil particles. Therefore the absence of detectable *Dehalococcoides* DNA over several site samples is suggestive (but not conclusive) that *Dehalococcoides* is absent.

6.3.2 Microcosm Testing

Prior to the development of molecular assays, microcosms containing site soil and groundwater were the only laboratory approach for assessing the presence of dehalorespiring microorganisms. Microcosm studies are still used in conjunction with molecular screening to determine the dechlorination activity associated with detected organisms, to determine degradation rates, acclimation periods, dechlorination products and optimization of electron donor type and dosing. Assessing the need to bioaugment a given site involves comparing the rate, extent, and acclimation period (time to initiate reductive dechlorination and achieve complete dechlorination to ethene) of dechlorination between non-bioaugmented and bioaugmented microcosms.

Electron-donor amended microcosms that do not dechlorinate PCE or TCE past *cis*-DCE after several months of incubation suggest that bioaugmentation is required. Microcosm testing services are provided commercially by several laboratories, including Shaw, BCI, Respirtek (www.respirtek.com) CL Solutions (www.cl-solutions.com) and SiREM.

6.3.3 Current Practices Based on Field Observations

There are several geochemical conditions that can be indicative of when bioaugmentation is likely to be required to enhance bioremediation of chloroethenes in groundwater. These include:

1. Sites with little or no evidence of anaerobic redox processes (*i.e.*, nitrate, iron, manganese or sulfate reduction). Such conditions likely will not have provided the opportunity for *Dehalococcoides* (which is strictly anaerobic) to become established;
2. Sites where biodegradation via reductive dechlorination of PCE and TCE to *cis*-DCE is occurring, but where VC and ethene are not detected at significant concentrations relative to parent products and *cis*-DCE. Of note, some production of VC and ethene can occur through either abiotic or anaerobic co-metabolic reactions. Accordingly, the presence of trace amounts of VC and ethene in groundwater samples should not be used to infer that complete dechlorination by anaerobic microorganisms is necessarily occurring unless supported by additional lines of evidence (*e.g.*, microcosm studies, molecular screening); and
3. Sites where the production of VC or ethene is not observed within a reasonable time frame after establishing appropriate redox/geochemical conditions. While quantification of “reasonable” is subjective, a period of six to nine months of electron donor addition should allow for an increase in an initial *Dehalococcoides* population of 10^2 cells/L groundwater (a cell concentration near or below typical detection limits) to 10^7 cells/L (a cell concentration which correlates with the detection of ethene at field sites) based on an assumed doubling time of 15 days. While longer electron donor periods can be pursued, the cost of long-term ineffective electron donor addition is likely to far exceed the cost of an early bioaugmentation.

6.4 Survival of Injected Organisms

The ability of an exogenous organism to survive and compete for resources against indigenous organisms has long been an area of interest in both macro and microbial ecology, and in many cases, added organisms do not fare well. For example, when three well-characterized toluene degrading bacteria, *P. putida* PaW1, *B. pickettii* PKO1, and *B. cepacia* G4 were added to a fluidized bed bioreactor with toluene as a feed source, strain PaW1 became the predominant organism in the reactor (Massol-Deya *et al.*, 1997). When groundwater strains were allowed to enter the reactor, however, even strain PaW1 was rapidly replaced. Thus, even pre-colonization of the reactor with the added strain did not prevent it from being rapidly displaced by native microbes.

An area that has not been well investigated is the selection or development of organisms with an inherent advantage for long-term survival, or that can be provided with an environment that

gives them a selective advantage. For example, CT degradation by *Pseudomonas* sp. strain KC was found to be greater under denitrifying conditions than under aerobic conditions (Criddle *et al.*, 1990; Lewis and Crawford, 1993). Furthermore, degradation was inhibited by dissolved iron. Through adjustment of the pH of growth media to 8.3, conditions under which iron is precipitated, this inhibition was overcome (Tatara *et al.*, 1993). Thus, strain KC had a competitive advantage over other aquifer organisms if grown under nitrate-reducing conditions at high pH. By adjusting the pH and redox potential of a contaminated aquifer, one could create a selective niche for added strain KC. These characteristics allowed Criddle and co-workers (Dybas *et al.*, 1998) to demonstrate the use of bioaugmentation for the remediation of a CT-contaminated aquifer in School Craft, Michigan.

In a related approach, Lajoie and colleagues (1992) isolated a bacterial strain, termed “field application vector”, that was resistant to a surfactant that it could also use as a carbon source. By cloning degradative genes into the resistant organism (making it a GEM), the researchers could add surfactants to an environment to create a selective niche, and then add the resistant strain containing the degradative genes (Lajoie *et al.*, 1994). Such strains may have utility for use in bioaugmentation of aquifers during or after surfactant or foam flushing designed to remove free product contaminants (Okuda *et al.*, 1996; Pennell *et al.*, 1996). However, the use of GEMs for bioaugmentation in aquifers is unlikely to occur in the near future due to regulatory constraints and adverse public perception of GEMs.

Bacterial predation by protists has been cited as a factor that could potentially limit the effectiveness of bioaugmentation. Protistan grazing on bacteria was assessed during a field-scale bacterial transport experiment in an Atlantic coastal plain aquifer (Choi *et al.*, in prep). It was estimated that protist grazing accounted for approximately 5% of the transported bacteria. However, protists were not abundant until near the end of the experiment, therefore, it was hypothesized that a much larger percentage of a second injection of bacteria would be subject to predation. Therefore, at sites where multiple injections of bacteria are necessary, predation could be a significant factor affecting survival of injected bacteria. Of note, this predation experiment was conducted in a pristine aquifer. Contaminated aquifers often have concentrations of protists several orders of magnitude higher than uncontaminated environments (Novarino *et al.*, 1997). Predation may therefore be more significant at sites that are candidates for bioaugmentation.

The fact that there are many potential reasons suggesting that introduced organisms may not fare well raises the question of the competitive advantages *Dehalococcoides* organisms might have in subsurface environments. The *Dehalococcoides* are efficient users of hydrogen and are capable of out-competing other organisms (*e.g.*, methanogens) for hydrogen (Fennell *et al.*, 1997; Duhamel *et al.*, 2004). Furthermore, *Dehalococcoides* tolerates concentrations of chloroethenes high enough to inhibit methanogenesis (Duhamel *et al.*, 2004). The apparently unique ability of *Dehalococcoides* to utilize DCE and VC as electron acceptors offers these organisms an otherwise-unoccupied metabolic niche. The combination of these advantages allows these organisms to compete with other subsurface organisms at chlorinated solvent sites and colonize the subsurface upon bioaugmentation. There are multiple examples of successful introduction, dechlorination and growth indicating that *Dehalococcoides* successfully competes with indigenous microorganisms. For example Major *et al.* (2002) demonstrated that the number of *Dehalococcoides* increased at least 10-fold due to growth after bioaugmentation.

Not only can introduced organism grow they can also persist sometimes for extended periods under adverse conditions. In 2002, GeoSyntec completed an *in situ* bioremediation field demonstration for SERDP (CU-1164) in which bioaugmentation with KB-1TM promoted rapid and complete dechlorination of TCE (2,000 µg/L) to ethene in an aquifer where dechlorination otherwise stalled at *cis*-DCE. One year following completion of the demonstration, groundwater samples were collected from monitoring wells in the test area for 16S RNA analysis, and *Dehalococcoides* microorganisms were detected in the test area despite the absence of electron donor addition for 12 months (Cox *et al.*, 2002). Recently (February 2004), groundwater from the same wells (collected two years following cessation of electron donor addition) contained biomass capable of supporting complete TCE dechlorination to ethene. It is speculated that biomass decay into fermentable organic compounds may prolong the activity of the dechlorinating organisms.

6.5 Microbial Transport in Groundwater

Bioaugmentation can be effective without significant bacterial transport if the remedial design calls for creating a relatively static biobarrier through which contaminated groundwater flows. This proved to be a very effective strategy for remediation of CT in groundwater at the Schoolcraft, Michigan site (Dybas *et al.*, 1998). This approach is particularly applicable at sites where the source of the contamination cannot be directly treated. The drawback to this approach is that it may take a considerable period of time before all of the contaminated groundwater passes through and is treated in the biobarrier.

At many sites, it is desirable to treat the entire contaminant plume over a short period of time. In these cases it is essential that an effective concentration of microorganisms be transported throughout the area of subsurface contamination. Efficient transport and dispersion of bacteria in the subsurface can significantly reduce the cost of bioaugmentation by reducing the number of injection points, as well as the time that it takes to impact and remediate the entire volume of contaminated groundwater. The design of remediation systems dependent on bacterial transport requires an understanding of the particular biological, physical and geochemical conditions that affect transport.

As summarized in Table 5, microbial transport in the subsurface depends on a number of characteristics of the cells themselves and the environment into which they are introduced. The highly complex interactions between physicochemical and biological processes affecting transport in part explains the inconsistencies present in the literature regarding the effect of bacterial properties on transport, and prevents making direct correlations between these properties and transport of the organisms.

Laboratory studies have defined a number of factors that can affect bacterial transport. These include the physicochemical factors such as grain size, flow velocity, solute chemistry and mineral surface charges, as well as the biological factors such as cell size, motility and chemotaxis, growth, and cell surface properties (charge, hydrophobicity, extracellular polymeric substances; Fontes *et al.*, 1991; Warren *et al.*, 1992; Weiss *et al.*, 1995; Harvey *et al.*, 1997; Ginn *et al.*, 2002). The results of field-scale bacterial transport studies have shown, however, that the data obtained from laboratory column studies may not adequately predict bacterial transport, even when intact core from the field site is used to simulate field transport (Scheibe *et al.*, 2001;

Table 5: Factors Impacting Microbial Transport And Growth

Factor	Impact on Microbial Growth & Survival	Impact on Microbial Transport	References
Temperature	Increases in temperature increase the rate of microbial growth and metabolic activity	Increased growth enhances transport	Jenneman et al., 1985; Reynolds et al., 1989; Sharma et al., 1993
pH	pH must remain within tolerance range for degradative microorganisms	Ionization of mineral grains and coatings can enhance transport at high pH	Scholl & Harvey, 1992; McEldowney & Fletcher, 1988
Ionic strength	Microbially activity unimpacted within typical range of ionic strength for groundwater	In general increased ionic strength reduces electrostatic repulsion and inhibits transport by increasing adhesion	Scholl et al., 1990; Simoni et al., 2000
Grain-size Distribution	Soil particles provide sites for biofilm growth	Enhanced migration in coarse soil; surface charge effects of clay fraction may retard particle migration. Size-exclusion may enhance transport	Harvey et al., 1989; Mayotte et al., 1996
Soil Mineralogy	Unknown; some minerals can provide limiting nutrients (Fe, P)	Metal oxyhydroxide coatings may retard transport due to charge interactions	Rogers (2002); Fletcher & Loeb, 1979; Scholl et al., 1990
Nutrient Availability	Bioavailable dissolved organic substrates may be utilized metabolically for cell growth and division	Nutrient addition increases aqueous-phase bacteria and enhances transport by growth	Murphy et al., 1997; see refs under temperature above
Groundwater Velocity	Minimal	Increasing groundwater velocity generally favors increases in cell detachment rates; higher velocity also decreases attachment for non-motile cells	Camesano and Logan, 1998; McCaulou et al., 1995
Bacterial Cell Surface	Multiple effects depending on the cell surface characteristic, the geochemistry of the environment and the mineralogy of the sediment matrix	Cell surface characteristics such as surface charge, surface polymers and proteins; hydrophobicity; flagella & pili affect adhesion properties and therefore transport	Sharma et al., 1985; Camesano & Logan, 2000; Caccavo, 1999; DeFlaun et al., 1990; Ginn et al., 2002
Bacterial Motility	Motility enables bacteria to move to favorable environments enhancing growth and survival	Motile bacteria exhibit chemotaxis and have the ability to enhance their transport over non-motile strains	Barton & Ford, 1999; Jenneman et al., 1985; Reynolds et al., 1989
Inoculum Cell Density	Higher cell densities provide more opportunity for bioaugmented biomass to colonize a favourable environmental niche	Higher inoculum densities may cause more attachment if cell-to-cell interactions are more favorable than cell-surface; if not favorable may enhance transport by blocking	Camesano and Logan, 1998; Ginn et al., 2002; Warren et al., 1992

Mailloux *et al.*, 2003). In general, laboratory column studies under-predict the extent of transport in the field. This is due in part to the three-dimensional aspect of field transport compared to the one-dimensional transport observed in column studies, as well as the length-scale of geologic heterogeneities. However, this disparity is also due to bacterial metabolism related effects and their temporal variations. Biological variability is often not obvious in short time-scale laboratory studies and the physical effects are not revealed due to the size of the cores relative to the field.

The effect of small-scale physical heterogeneity on bacterial transport has been observed in field experiments. Harvey *et al.* (1993) simultaneously injected bacteria, microbial-sized microspheres and bromide in a sandy aquifer on Cape Cod, MA. The relative order of breakthrough differed for three sample locations in a single well within one meter of the injection point. Similar observations were made in a relatively uniform sandy aquifer with maximum variations in hydraulic conductivity of approximately one order of magnitude (Hubbard *et al.*, 2001). The breakthrough of bacteria in two multi-level sampler ports separated vertically by only 27 cm, varied significantly in both magnitude and the timing of peak breakthrough (Mailloux *et al.*, 2003). However, the range in bacterial surface properties, even in the pure culture injected, was great enough to mask the effects of aquifer heterogeneity on overall transport.

The biological factors that govern transport are the primary reason why bacterial interactions with solid surfaces are inadequately described by DLVO theory of colloid stability and why models relying on colloid filtration theory are inadequate to describe bacterial transport (Ginn *et al.*, 2002). In a number of field experiments the majority of the introduced bacteria do not travel very far from the injection point (Harvey, 1997; DeFlaun *et al.*, 1997), most likely due to adhesion to mineral surfaces. This effect can be exacerbated by cell-to-cell interactions, which if favorable can increase subsequent attachment near the injection point.

In cases where bacteria have been observed to travel much farther than predicted, a number of mechanisms related to attachment and detachment from sediment surfaces have been invoked to explain this phenomenon. Transport data from several field studies have reflected either a bimodal or a continuum of cell surface properties within a monoclonal population that results in a range of attachment rates (DeFlaun *et al.*, 1997; Mailloux *et al.*, 2002; Schijven *et al.*, 2000). In addition to exhibiting a range of 'stickiness' that contributes to far-field transport, detachment can also contribute significantly to transport over longer distances. This detachment may occur as a result of physical processes, such as hydrodynamic shear; however, active biological detachment and growth are significant factors.

Increases in aqueous phase bacteria have been observed with substrate addition, which may reflect *in situ* growth (Jenneman *et al.*, 1985; Murphy *et al.*, 1997), although in some cases it has been attributed not to growth, but the change in the chemistry of the groundwater causing bacterial detachment (Mailloux and Fuller, 2003). The importance of growth in mediating transport was observed in a bioaugmentation field demonstration of chlorinated ethene degradation (Major *et al.*, 2002). Only a small inoculum (13 L) of the dechlorinating consortium was injected to treat a pore volume of approximately 64,000 L. Members of the consortium were observed to grow throughout the pilot test area (9.1 m long x 6.3 m wide) within 142 days of injection and increased in concentration with time. The total number of cells in the pilot test area

at the end of the test was significantly greater than the original inoculum. For more adhesive cells that do not remain in the aqueous phase, growth-related transport represents an effective transport mechanism.

6.6 Field Application of Bioaugmentation

Detailed descriptions of several bioaugmentation field demonstrations are provided in Attachment C. Summaries of available peer-reviewed and gray literature bioaugmentation demonstrations are presented in Table 6. While the majority of these demonstrations have employed mixed consortia, four studies have employed pure cultures utilizing aerobic cometabolic biodegradation processes. These cometabolic studies resulted in significant decreases in the concentrations of the target contaminants, although, as discussed previously, the applicability of this technology appears limited.

Many contaminated aquifers are either naturally anaerobic or are anaerobic due to the degradation of contaminants. In the presence of sufficient electron donor, aerobic and facultative bacteria deplete oxygen resulting in a reducing environment suited to anaerobic bioaugmentation. To date, twelve field demonstrations of anaerobic bioaugmentation, most of which significantly enhanced the extent of dechlorination to ethene, have demonstrated the effectiveness of this approach although there have been no reported cases in the literature where the technology has been used successfully to achieve site closure. Many aspects of the technology are still in the developmental stages; however, the database of successful demonstrations, as well as continued data collection at older test sites, has provided an improved understanding of the parameters that control the success of bioaugmentation.

Enhanced bioremediation systems employing bioaugmentation typically consist of an electron donor delivery system and a means of adding the dechlorinating biomass into the subsurface. As shown in Table 7, a range of electron donor delivery strategies, including both semi-passive and active approaches, have been employed. In each case, mixing of the contaminants, nutrients and microorganisms are essential for achieving remediation. Many of the demonstrations have used active groundwater recirculation systems for electron donor amendment, although this is due to the short-term research nature of these studies and the desire to control variability in groundwater flow.

As shown in Table 7, delivery systems for bioaugmenting groundwater with dechlorinating biomass generally involve either: 1) continuous injection of dechlorinating biomass (low cell densities) contained in either site groundwater or the effluent of anaerobic bioreactors initially seeded with dechlorinating biomass (two demonstrations); or 2) batch injection of dechlorinating biomass contained within an enriched culture (high cell densities; thirteen demonstrations). While the use of enriched cultures appears to be the typical practice, recirculation of groundwater containing dechlorinating biomass may be a low-cost approach to providing the requisite biomass, although the performance of this technique is not well-understood. Reported cell densities of batch-injected cultures range from 10^8 to 10^{11} cells/mL with the biomass typically suspended either in site groundwater or within a reduced growth media.

Table 6: Summary of Bioaugmentation Field Demonstrations for Chlorinated Solvents in Groundwater¹

	Contaminants	Demonstration Location	Geologic Setting	Bioaugmentation Culture	Amendments	Key Findings Relevant to Field Performance	Reference/Source
Aerobic	TCE, DCE, & VC	Gilbert-Mosley Site, Wichita, KS	Sand	<i>Burkholderia cepacia</i> PR1 ₃₀₁	Dissolved oxygen	Reduction of total chloroethene concentration from 250 ug/L to non-detect within 24 hours (maintained for 4 days).	Bourquin et al. (1997)
	TCE	Industrial Facility, Pennsauken, NJ	Silty, fine to medium sand with clay lenses	<i>Burkholderia cepacia</i> ENV435	Dissolved oxygen (up to 20 mg/L)	TCE degradation was observed within several days. Cell densities remained high during the 30 day study. Estimated removal of degradable VOCs ranged from 44 to 78%.	Steffan et al. (1999)
	TCE	Flemington, NJ	Moderately permeable weathered bedrock	<i>Burkholderia cepacia</i> ENV435	Dissolved oxygen	Average TCE concentrations in monitoring wells were reduced by 67 to 94%.	Walsh et al. (2000)
	TCE	Chico Municipal Airport, Chico, CA	Cobbles and finer-grained materials	<i>Methylosinus trichosporium</i> OB3b	Higgins phosphate buffer (10 mM)	Over the first 50 hours, TCE concentrations decreased from 425 ppb to <10 ppb. TCE concentrations gradually increased to background levels over 40 days.	Duba et al. (1996)
Anaerobic	PCE, TCE, 1,1,1-TCA, chloroform	Caldwell Trucking Superfund Site, NJ	Fractured bedrock	KB-1	Weekly addition of methanol, lactate and acetate	Decreases in TCE concentration as high as 90%; only slow conversion of cis-DCE to VC and ethene (ongoing)	Finn et al. (2003)
	PCE & TCE	Evenblij Site, Hooeven, the Netherlands	Sand	On-site anaerobic bioreactors inoculated with sludge from an industrial upflow anaerobic sludge blanket reactor	Periodic addition of acetate and lactate (200 mg/L as DOC)	Complete degradation of PCE to ethene was achieved within 28 days after delivering bioreactor effluent. Effluent was filtered to reduce solid density and prevent well fouling prior to delivery. Well fouling was further prevented by regeneration with citric acid once weekly. Citrate also served as an electron donor.	Henssen et al. (2001)
	TCE	Cape Canaveral AFS, FL	Fine to medium sand, silt, and shells	KB-1	Pulsed injection (1 hour per day) of 520 mg/L EtOH (TWA)	Biostimulation enhanced dechlorination rates although only minimal conversion to ethene observed. Subsequent to bioaugmentation, complete and rapid conversion to ethene occurred throughout the test plot.	MacMaster et al. (2002)
	TCE	Dover AFB, DE	Fine sand and silt	Pinellas	Continuous recirculation with lactate (100 mg/L), ammonia (5 mg/L) and phosphate (5.5 mg/L)	Following bioaugmentation, a 90 day lag occurred before dechlorination of cis-DCE was observed. Complete conversion of TCE and cDCE to ethene was achieved.	Ellis et al. (2000)
	TCE	Aerojet Superfund Site, Sacramento, CA	Unconsolidated fluvial deposits containing sand and gravel	KB-1	Pulsed injection (1 hour per day) of lactate (50 mg/L TWA)	Some TCE dechlorination to cis-1,2-DCE was observed through lactate addition alone. Following bioaugmentation with KB-1, VC and ethene production from cis-1,2-DCE were observed within 8 days; chloroethene concentrations decreased below criteria 15 feet from the electron donor delivery well within 125 days.	Cox et al. (2000)

Table 6: Summary of Bioaugmentation Field Demonstrations for Chlorinated Solvents in Groundwater¹ (continued)

	Contaminants	Demonstration Location	Geologic Setting	Bioaugmentation Culture	Amendments	Key Findings Relevant to Field Performance	Reference/Source
Anaerobic	TCE	Industrial Facility, Boston, MA	Unconsolidated fluvial deposits underlain by glacial outwash	KB-1	Weekly pulsed addition of acetate (100 mg/L) and methanol (500 mg/L)	Bioaugmentation resulted in production of ethene within one month (following four months of electron donor addition). Sulfate(>1,500 mg/L) did not inhibit reductive dechlorination. Stable carbon isotope analysis confirmed that biodegradation was occurring.	Chang et al (2002); Chang et al (2003)
	PCE & Carbon Tetrachloride	Dow Facility, Pittsburgh, CA	Unconsolidated fluvial/alluvial deposits (clay, silt, sand & gravel)	Site groundwater	Periodic addition of sodium lactate (10%) and ammonium polyphosphate (56%)	Flux of VOCs reduced by 21% (weighted avg. based on reported data); increase in conversion to ethene. Degradation rate of PCE increased by 6X; degradation rate of carbon tetrachloride increased by 3X.	Jin et al. (2002); Droy et al. (2002)
	PCE	Kelly AFB, TX	Unconsolidated alluvial deposits	KB-1	Daily pulsed injection of methanol and acetate (time-weighted average concentrations of 3.6 mM each)	Bioaugmentation of the test plot resulted in complete transformation of PCE to ethene after a lag period of ~70 days.	Major et al. (2002)
	PCE	Dover AFB, DE	Fine sand and silt	KB-1	Daily injection of ethanol (18 mg/L TWA) and lactate (69 mg/L TWA)	Dechlorination of PCE to cis-DCE after 164 day of electron donor addition; 32 days after bioaugmentation, minimal impacts have been observed (study currently in progress).	MacMaster et al. (2002)
	PCE	Industrial Facility, Chester, SC	Fractured metagabbro	KB-1	Daily pulse injection of methanol (160 mg/L TWA) and lactate (25 mg/L TWA)	Conversion of conversion of PCE to cis-DCE; repeated bioaugmentation resulted in partial dechlorination to ethene (~30%).	GeoSyntec unpublished data; Konzuk (2002)
	PCE	Bachman Road Residential Wells Site, MI	Fine to medium grained sand	Bachman Road culture (Bio-Dechlor)	Continuous recirculation with lactate (0.1 mM), phosphate, and nitrate.	Bioaugmentation resulted in complete conversion of PCE to ethene in six weeks. <i>Dehalococcoides</i> increased by up to 4 orders of magnitude in the bioaugmentation plot. Complete dechlorination was only observed after a three month lag in the biostimulation plot.	Lendvay et al. (2003)
	Carbon Tetrachloride	Schoolcraft, MI	Glacial outwash sands			Bioaugmentation resulted in the biodegradation of carbon tetrachloride to carbon dioxide without production of chloroform.	Dybas et al. (1998); Dybas et al. (1997)

Notes

¹ Data presented are derived from peer-reviewed academic publications, technical reports, and abstracts presented at conferences.

TCE - trichloroethene

PCE - tetrachloroethene

DCE - dichloroethene

VC - vinyl chloride

TEX - toluene, ethylbenzene and xylene

Table 7: Summary of Bioaugmentation Field Protocols

Demonstration Location		Bioaugmentation Culture	Innoculant Volume	Estimated Cell Density	Description of Bioaugmentation Protocol
Planktonic Biomass ¹	Dow Facility, Pittsburgh, CA	Site groundwater	Up to 900 gallons per event	10 ⁴ -10 ⁶ cells/mL (as determined by nitrate reducer MPN)	Injection of site groundwater from areas of high dechlorinating activity through groundwater recirculation wells.
	Evenblij Site, Hoozeveen, the Netherlands	Groundwater containing dechlorinating biomass	13 x 10 ⁶ L of bioreactor effluent (filtered prior to infiltration)	Not reported (suspended solids <0.5 mg/L)	Filtered effluent from an on-site anaerobic bioreactors inoculated with sludge from an industrial upflow anaerobic sludge blanket reactor was infiltrated into groundwater.
Enriched Cultures or Mixed Consortia	Gilbert-Mosley Site, Wichita, KS	<i>Burkholderia cepacia</i> PR1 ₃₀₁	Not specified	10 ⁹ cells/mL (in situ)	Standing water in the injection well was recirculated (top to bottom) 19 times to provide mixing.
	Pennsauken, NJ	<i>Burkholderia cepacia</i> ENV435	550 L	1.2 x 10 ¹¹ CFU/mL	Culture was metered into an injection well at ~3 L/min over ~3.7 hours. The groundwater recirculation rate was 3 L/min.
	Flemington, NJ	<i>Burkholderia cepacia</i> ENV435	150 gallons	5 x 10 ⁸ cells/mL (in situ)	178 gallons total (bacteria + carbon source + water) was pneumatically injected into four discrete fractured zones in one well in fractured bedrock.
	Chico Municipal Airport, Chico, CA	<i>Methylosinus trichosporium</i> OB3b	1,800 L	5.4 x 10 ⁹ cells/mL	Culture injected into a single well at 3.8 L/min over 7.9 hours, followed by uncontaminated groundwater (400 L) amended with Higgin's phosphate buffer (10 mM).
	Schoolcraft, MI	<i>Pseudomonas stutzeri</i> KC	1,500 L	Not reported	Inoculum was pumped into an injection well at 189 L/min
	Bachman Road Residential Wells Site, MI	Bachman Road culture (Bio-Dechlor)	200 L	1.12 x 10 ⁸ cells/mL	a. Groundwater (75 L) was amended with inoculum (200 L) and sulfide (5 mg/L); 220 L of suspension was injected into the test plot.
					b. Groundwater (110 L) was mixed with the remaining suspension; 128 L of diluted suspension was injected into the test plot.
					c. Groundwater was amended with lactate (0.1 mM) and nutrients (phosphate, nitrate) after bioaugmentation.
	Dover AFB, DE	Pinellas	180 L and 171 L	<2x10 ⁸ cells/mL	a. On Day 269 of substrate injection, 180 L of culture was injected into the operating injection well under a nitrogen blanket.
					b. On Day 284 of substrate injection, 171 L of culture was injected into the operating injection well under a nitrogen blanket.
					c. During both events, a surge block was positioned at the top of the screen to ensure immediate delivery of the culture into the aquifer.
					d. Substrate addition was resumed 24 hours after bioaugmentation.

Table 7: Summary of Bioaugmentation Field Protocols (continued)

Demonstration Location		Bioaugmentation Culture	Innoculant Volume	Estimated Cell Density	Description of Bioaugmentation Protocol
Enriched Cultures or Mixed Consortia	Cape Canaveral AFS, FL	KB-1	40 L	$\sim 10^8$ cells/mL (<i>Dehalococcoides</i> only)	Each of the three injection wells was purged with argon. Equal portions of culture were injected below the watertable under positive argon pressure.
	Aerojet Superfund Site, Sacramento, CA	KB-1	50 L	$\sim 10^8$ cells/mL (<i>Dehalococcoides</i> only)	Culture was injected below the watertable through a monitoring well under positive argon pressure
	Industrial Facility, Boston, MA	KB-1	40 L	$\sim 10^8$ cells/mL (<i>Dehalococcoides</i> only)	Culture was injected below the watertable into a groundwater recirculation well under positive argon pressure
	Kelly AFB, TX	KB-1	13 L	$\sim 10^8$ cells/mL (<i>Dehalococcoides</i> only)	a. Groundwater recirculation was halted and the injection well purged with argon. Culture injected below the watertable under positive argon pressure.
					b. Recirculation briefly restarted (three casing volumes) to provide culture with electron donors.
					c. Recirculation was halted for 24 hours to permit bacteria to establish in the subsurface.
	Dover National Test Site, DE	KB-1	55 L	$\sim 10^8$ cells/mL (<i>Dehalococcoides</i> only)	a. Groundwater recirculation was halted and the injection well was purged with argon. Culture (11 L) was injected below the watertable under positive argon pressure. The procedure was repeated for each of the three injection wells.
					b. Two test plot monitoring wells were amended with EtOH (360 mL) and groundwater (5 gal) and then purged with argon. Culture (11 L per monitoring well) was injected below the watertable under positive argon pressure.
	Industrial Facility, Chester, SC	KB-1	40 L for each bioaugmentation event	$\sim 10^8$ cells/mL (<i>Dehalococcoides</i> only)	a. In the first event, a monitoring well was purged with argon. Culture was injected below the watertable under positive argon pressure.
					b. For the second event, groundwater recirculation was halted and the injection well was purged with argon. Culture was injected below the watertable under positive argon pressure.

Notes

¹ Bioaugmentation of planktonic biomass is typically performed at cell densities comparable to those observed in groundwater environments (e.g. 103-105 cells/mL)

6.7 Emerging Practices: Bioaugmentation for DNAPL Source Zone Treatment

Chlorinated solvents such as PCE and TCE are present in groundwater as DNAPLs at many DoD, Department of Energy (DoE), and related contractor facilities. Unfortunately, the aqueous solubility of most DNAPLs is low enough that they dissolve slowly in groundwater and act as long-term sources of groundwater contamination, but high enough that dissolved concentrations exceed regulatory criteria by as much as five orders of magnitude (Pankow and Cherry, 1996). Given recent data demonstrating the activity of dechlorinating microorganisms at high VOC concentrations, bioaugmentation may enhance DNAPL dissolution rates while providing *in situ* degradation of the target VOCs since a significant increase in the biodegradation rate of the parent DNAPL can increase in the dissolution rate (*e.g.*, Seagren *et al.*, 2002; Yang and McCarty, 2000; Chu *et al.*, 2003). Dissolution enhancement factors in the rates of DNAPL removal from the results of bench-scale studies and modeling analyses are provided in Table 8.

To evaluate the impact of enhanced bioremediation on the rate of DNAPL removal at the field-scale and to develop design protocols for technology implementation in high concentration source areas, ESTCP is currently funding multiple projects evaluating the impact of biodegradation on DNAPL removal rates, including demonstrations at Dover AFB (Delaware), Cape Canaveral Air Force Station (Florida), and Fort Lewis (Washington). In each case, groundwater recirculation systems will be used to control electron donor delivery to the source area, and detailed monitoring is being performed to evaluate the impact of the biodegradation process on contaminant removal and degradation rates..

Recently, a pilot-scale technology demonstration employing bioaugmentation has been completed at Launch Complex 34, Kennedy Space Center, within a zone containing substantial TCE DNAPL with an average TCE concentration of 150 mg/L (McMaster *et al.*, 2003). While a native *Dehalococcoides* microorganism, tentatively identified as a member of the Pinellas sequence subgroup that is capable of complete TCE dechlorination, is present at the site only minimal conversion to ethene occurred following the addition of electron donor. Following bioaugmentation, rapid increases in the rate and extent of conversion to TCE to ethene were observed. A detailed description of this demonstration is provided in Appendix C.

Table 8: DNAPL Mass Transfer Enhancements Achieved using Enhanced Bioremediation

Reference	Study Description	Maximum Mass Transfer Enhancement
Carr <i>et al.</i> (2000)	Continuous-flow stirred tank reactors containing PCE/tridecane DNAPL	3
Yang & McCarty (2000)	Batch reactors containing PCE DNAPL	5
Cope and Hughes (2001)	Laboratory mesocosm containing PCE/tridecane DNAPL	6.5
Heidorn <i>et al.</i> (2002)	Laboratory mesocosms containing PCE DNAPL	2
Seagren <i>et al.</i> (2002)	Laboratory mesocosm containing toluene/dodecane LNAPL	1.87
Adamson <i>et al.</i> (2003)	Laboratory mesocosm containing PCE DNAPL	1 [†]
Chu <i>et al.</i> (2003)	Modeling analysis	~4

Notes

[†] Estimated based upon effluent mass flux (abiotic flux not measured)

7. REGULATORY CONSIDERATIONS

To date, more than 50 field-scale bioaugmentation projects have been conducted in at least 21 states, including Alaska, California, Colorado, Delaware, Florida, Georgia, Indiana, Kansas, Massachusetts, Michigan, New Hampshire, New Jersey, New York, Ohio, Oregon, Pennsylvania, Rhode Island, South Carolina, South Dakota, Texas and Utah. Based on discussions with various bioaugmentation vendors and consultants who have applied bioaugmentation, it appears that few states have regulations or permit requirements pertaining specifically to the injection of bacteria into the subsurface.

Instead, the injection of microbial cultures has typically been covered under the same permitting process as for injection of bioremediation nutrients (*e.g.*, electron donors). The specific type of permit required is largely dependent on oversight authority, with federal (*e.g.*, USEPA) projects typically requiring an Underground Injection Control (UIC) permit, and state-led projects requiring either a state-UIC permit or equivalent (*e.g.*, Waste Discharge Requirements [WDRs] in California).

8. INFORMATION AND RESEARCH NEEDS

The results of multiple controlled field demonstrations are beginning to show the potential significant benefits of bioaugmentation as a tool to improve the success of enhanced bioremediation applications. However, there are still a number of technical questions that remain to be answered to improve the design, implementation and efficacy of bioaugmentation applications. This section identifies and discusses research and information needs for bioaugmentation, in terms of culture production and QA/QC, and technology design, implementation and performance validation.

8.1 Culture Production and Quality Assurance/Quality Control

Production of mixed microbial cultures requires an ongoing QA/QC program to prevent the introduction of undesirable microorganisms and to ensure continued activity and numbers of active organisms. It is recommended that the following information be provided by the vendors prior to use of any inoculum:

- Pathogenicity: Given potential concerns regarding safe handling and injection of microbial cultures into groundwater supplies, and the potential human health impacts resulting from pathogen exposure, pathogenicity data should be required for all cultures prior to use at DoD and related contractor facilities. Furthermore, since the community composition of these microbial cultures is likely to evolve over time (in response to enrichment and handling), rigorous QA/QC procedures designed to maintain culture stability and avoid inadvertent pathogen introduction should be in place. Regular testing should be conducted to confirm the absence of pathogenic organisms.
- Culture Composition: To the best of our knowledge, each of the microbial cultures in use for anaerobic bioaugmentation is a mixed consortium enriched from a VOC-impacted site. The microbial community composition of these cultures likely varies, as do the degree of enrichment and the availability of information regarding the community composition. Ultimately, it would be beneficial to enrich these cultures so that they contain only those organisms required for effective application.
- Culture Efficacy: As indicated above, the community composition of the microbial cultures is likely to evolve over time in response to enrichment and handling, and therefore, analysis of community composition and degradation performance should be conducted periodically, and data should be available for review to ensure that the culture and its efficacy is similar to that of culture batches for which vendor claims are being made.
- Culture Handling: Vendors should specify on culture shipping containers whether or not VOCs may be present in the culture. Further culture labeling should meet relevant standards (e.g., USDOT, OSHA). Based on the limited information available, it appears that a variety of containers, ranging from plastic buckets to anaerobic stainless steel vessels are used for culture shipment. It is unclear to

what extent culture handling practices affect culture survival during storage shipment, and introduction to the subsurface. Investigations should be conducted to compare handling practices, so as to determine “best-practices” for use at DoD sites, which will in turn improve the chances for success of bioaugmentation.

- Culture Storage: There is currently very little information available regarding culture storage shelf-life. Testing should be conducted to evaluate the effects of storage and starvation on cultures being used or considered for use, so as to assist in developing “best-practices” for use at DoD sites.

8.2 Technology Design, Implementation & Performance Validation

To date, bioaugmentation has been conducted using a variety of designs and infrastructure, including both direct injection and recirculation approaches. However, despite the increasing number of bioaugmentation applications, there is still very little known regarding the effectiveness of current bioaugmentation protocols. The development of improved bioaugmentation field protocols (*e.g.*, injection technique, injection rate, cell density, need for electron donor amendment) will likely lead to improved success of this technology. Furthermore, research is required to evaluate the following issues:

- Aquifer Pre-Conditioning: Current bioaugmentation practices favor a period of aquifer pre-conditioning, whereby nutrients (most commonly electron donors) are added to the aquifer to establish the desired redox conditions (through the activity of indigenous bacteria) prior to bioaugmentation with the subject culture (Ellis *et al.*, 2000; Major *et al.*, 2002). In many cases, the pre-conditioning phase is also used to assess the degradation potential of the indigenous bacteria, and to confirm the need for bioaugmentation. Unfortunately, there is little information available regarding optimization of these pre-conditioning activities. If the pre-conditioning step is insufficient, the activity of the bioaugmented organisms may be adversely impacted. If the pre-conditioning step is too long, it is possible the bioaugmented culture may suffer from increased competition and/or predation by non-beneficial organisms. Research studies comparing culture establishment and activity under controlled site conditions with varying periods of aquifer pre-conditioning may be highly beneficial in optimizing bioaugmentation performance and cost.
- Culture Requirements: To date, most field applications of bioaugmentation have involved small-scale demonstrations, or small sites (see for example Ellis *et al.*, 2000; Major *et al.*, 2002), where the amount of organism added has a minimal effect on treatment costs. In these cases it may not be necessary to optimize the amount of culture added. However, on large sites where large volumes of culture are required, knowing the minimum amount of culture needed for successful remediation could result in considerable cost savings. Research is needed to evaluate the amount of culture needed for optimum remedial activity under different geophysiochemical conditions including porosity, contaminant concentration, and temperature.
- Delivery Methodology: To date, some of the bioaugmentation field applications that have been demonstrated to be highly effective have employed recirculation

systems (rather than direct injection) where the organisms were readily added and distributed. Several of these studies (Ellis *et al.*, 2000; Major *et al.*, 2002; Cox *et al.*, 2002) demonstrated transport, establishment and growth of dehalorespiring bacteria within the environment. Unfortunately, there is little peer-reviewed information regarding the establishment and growth of bacteria introduced through direct injection, although concerns have been raised regarding the potential impact of injection pressures on culture survival. Furthermore, direct injection approaches have typically required sequential injection of donor and organisms, which raises questions as to effective mixing of these required constituents. It is also unclear whether injection of the organisms with high concentrations of slow-release electron donors may inhibit or benefit organism establishment and activity. Research evaluating the establishment, growth and activity of organisms introduced into the subsurface via direct injection techniques will help validate this approach.

- *In Situ* Transport and Distribution: Significant questions remain regarding the transport of the various microorganisms in bioaugmentation cultures in the subsurface. While several field demonstrations have shown that *Dehalococcoides* are relatively mobile, there is little to no information available describing the transport of other organisms contained in the bioaugmented consortia. Furthermore, there is little information available as to the spatial distribution of dechlorinating activity achieved at the test sites. Specifically, while *Dehalococcoides* have been reported to travel to distances of several hundred feet from their point of introduction, it is unclear whether the transport of this organism was accompanied by a corresponding increase in dechlorination activity. This information has important ramifications with regards to design of bioaugmentation systems and in particular spacing of bioaugmentation delivery points. Collection of this information is the subject of an ongoing ESTCP project (CU-0315).
- Electron Donor Addition & Survival: A variety of electron donors and feeding strategies have been employed for bioaugmentation projects. However, there is currently little understanding regarding the impacts of varying electron donor addition strategies on survival and activity of the introduced cultures. Research has shown that controlled release of electron donors may favor the establishment and activity of dehalorespiring bacteria, versus the “over-feeding” approaches more commonly employed in bioremediation projects. Furthermore, emerging field evidence is showing that degradation activity can be maintained by biomass decay (Cox *et al.*, 2002) therefore, it may be possible/beneficial to incorporate this feature into electron donor delivery strategies, to periodically reduce unwanted biomass, and to reduce electron donor and operational costs. Further research on electron donor optimization strategies would likely improve bioaugmentation and bioremediation applications at DoD sites.
- Effects of Timing of Augmentation: Bioaugmentation has been viewed as a “prophylactic” insurance measure or as a contingency if biostimulation fails. In some cases, systems may be operated for 6-18 months before complete dechlorination occurs. However, waiting to augment until biostimulation systems

have operated for several months entails a risk similar to that described earlier for aquifer preconditioning (i.e., other organisms may have become established, leading to increased competition and/or predation that may make the added beneficial organisms less effective). To date, we do not have any comparative demonstrations or controlled experiments that would allow us to judge whether such theoretical concerns are justified.

- Performance Monitoring & Validation: Bioaugmentation projects should continue to incorporate quantitative techniques for the enumeration of introduced organisms, to help improve understanding of the establishment, growth, activity and survival of introduced organisms. Furthermore, research would be beneficial to develop methodologies (e.g., DNA microarrays to assess gene expression of relevant pathways) to readily delineate the different phenotypes of *Dehalococcoides*, so that *Dehalococcoides* behavior can be predicted.

9. ACCELERATING TECHNOLOGY TRANSITION

Ensuring the appropriate application of a new technology requires significant efforts to inform site stakeholders as to the appropriate application of bioaugmentation. Clearly, bioaugmentation is not a novel technology, having been historically employed for wastewater treatment, pest control, and the remediation of hydrocarbon contaminated soil and groundwater, although the current trend toward the use of bioaugmentation for the remediation of chlorinated VOC contaminated groundwater may still be considered novel by some environmental practitioners.

In addition to overcoming the concerns of project stakeholders and the public, regulatory processes have a significant impact on the application of this technology. In situ bioremediation often requires the injection of substrates and bacteria, permitting for which is controlled by federal and/or state underground injection control regulations, and water quality criteria. A number of jurisdictions have groundwater criteria for common electron donors. For example, Florida regulates the concentration of methanol in groundwater at a concentration low enough to prevent the use of this electron donor.

Based on the experience gained through the development of bioaugmentation for hydrocarbon remediation and the relative ease with which dechlorinating cultures may be isolated, it is evident that the application of bioaugmentation for chlorinated VOC remediation requires meaningful standards of practice that protect the public by minimizing the potential for the introduction of pathogenic or opportunistic microorganisms based on the following questions:

- In the case of enriched indigenous cultures, are pathogens known to be present in the culture (determined via DGGE, pathogen-specific testing, etc.)?
- In the case of enriched indigenous cultures, is there a history of waste disposal or land use at the location from which the initial isolate was obtained that suggests that human or animal pathogens may be present?
- Do the methods of culture isolation, enrichment, maintenance, and monitoring employ procedures to ensure that pathogenic or opportunistic organisms are not inadvertently introduced?

At the same time, the development of accepted standards of practice are essential to ensure the effective application of this technology at the field-scale, including:

- The development of standard techniques and descriptors for monitoring the composition and dechlorinating activity of bioaugmentation cultures (*e.g.*, cell density, *Dehalococcoides* enumeration, CVOC dechlorination rates, *etc.*);
- Completion of well-monitored full-scale field demonstrations with results presented in the public domain for review to ensure that mixed cultures are effective and robust in the field; and
- Protocols for monitoring the performance of bioaugmentation at field sites such that the extent of dechlorination is readily apparent.

10. SUMMARY

Bioaugmentation has progressed from the realm of “snake-oil salesmen” selling unnecessary cultures into an ecologically credible and economically attractive technology. In particular, augmentation can be a useful option for improving the cleanup of chlorinated solvents, because *Dehalococcoides* capable of complete dechlorination are unique organisms that are present at many, but not all sites, and their numbers are often very low and their distribution within the subsurface is often patchy. Adding mixed cultures containing *Dehalococcoides* has proven to reduce the time needed for complete dechlorination to occur at many sites, and at some sites it appears to be essential for achieving complete dechlorination.

Although there is much we still do not know, there has been significant progress. Proven cultures are commercially available, and their value has been demonstrated under field conditions. Cultures can be grown efficiently, transported to field sites effectively, successfully injected, and in most cases they will survive and grow in aquifers given proper environmental conditions. Bioaugmentation appears to be compatible with most of the common electron donors, and has been used with lactic acid, molasses, vegetable oil and HRCTM.

The key issues appear to be determining *a priori* whether bioaugmentation will be beneficial, ensuring adequate distribution and concentration of added cultures throughout a target zone, overcoming potential inhibitory conditions, and keeping costs low while ensuring adequate quality assurance. Finally, the roles of the other organisms present within the mixed cultures used for inoculation are not clear. Other organisms appear to be needed for complete dechlorination, at least at some sites.

Project managers considering the use of bioaugmentation should address the issue as early in the design stage as possible. PMs should complete an explicit cost-benefit assessment, including a life-cycle cost analysis, to determine whether bioaugmentation has the potential to reduce the time, costs, and/or regulatory acceptance of a bioremediation approach. Site-specific testing should be performed to aid in making this decision, including the use of focused microbial analyses and molecular biological tools to identify the capability of microorganisms at a specific site to quickly achieve complete dechlorination.

In closing, it is important to stress that, if bioaugmentation is to be used, PMs should work closely with the culture vendors to ensure that the cultures are added in a manner that maximizes the potential for success. The timing and locations of injections, and the numbers of organisms added, should all be carefully designed, with the vendors included in the decision-making process. Finally, the aquifer must be preconditioned, and environmental factors must be monitored and controlled during and after injections, to ensure that the added organisms have the best possible opportunity to survive and thrive.

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APPENDIX A
BIOAUGMENTATION CULTURE VENDOR SURVEY

Dear Sir/Madam:

Given the increasing field application of bioaugmentation for the remediation of chlorinated solvents in groundwater, the Environmental Security Technology Certification Program (ESTCP) is supporting the preparation of a comprehensive technical paper that: 1) summarizes past and current bioaugmentation practices; 2) defines the current state of knowledge surrounding the microorganisms being used for bioaugmentation and their biodegradation mechanisms; 3) summarizes the results of the various successful bioaugmentation field demonstrations; and 4) summarizes anticipated information and research needs to facilitate technical and stakeholder (*e.g.*, regulatory) acceptance of bioaugmentation and optimize the benefit of this technology for use by the Department of Defense (DoD), government and industry. The technical paper is based on public-domain and peer-reviewed scientific literature, and will be reviewed by a number of technical experts, including the ESTCP Technical Panel and independent academics.

ESTCP has identified your firm as one of the commercial suppliers of a dechlorinating microbial culture and requests your cooperation with this effort by providing the following non-proprietary information. This information will be included in the technical paper, a copy of which will be available to DoD Remedial Project Managers and to the Public.

Culture Description

- 1) What is the name, identifier or reference number of your culture?
- 2) What is the original source of the inoculum used to commercially produce the culture (*e.g.*, VOC-impacted site, wastewater treatment plant, other)?
- 3) Is microbiological characterization information available for the culture? Please cite reference or provide information, if possible.

Culture Use

- 4) Has your the culture been injected in a field setting? If so, please list:
 - a. States where culture has been deployed.
 - b. Site conditions where culture has been deployed.
 - c. Rationale for bioaugmentation (*i.e.*, dechlorination stalled).
 - d. Method of delivery to site.
 - e. Method of delivery to aquifer (direct injection vs recirculation).
 - f. Volume of culture added and rationale.
- 5) Was aquifer redox acclimation required/conducted prior to bioaugmentation?
- 6) Were regulatory permits required for addition of organisms to the aquifer? If so, please list/describe.

- 7) Was the fate of the added culture/microorganisms tracked following delivery? If so, please state methods.

Production & Quality Assurance/Quality Control

- 8) What quality assurance/quality control (QA/QC) measures do you employ to:
- a. Ensure that the culture maintains consistent VOC degradation activity comparable to that of the original culture?
 - b. Verify that the density of dechlorinating biomass is uniform in successive batches of culture?
 - c. Assess and verify the stability of the microbial community within the culture over time and production lots?
 - d. Ensure that the culture is free of pathogenic, opportunistic or other microorganisms that may be undesirable in terms of culture performance or safety concerns?

While we recognize that the specific protocols used for commercial production and testing of these products may include proprietary or commercially-sensitive information, the objective of this technical review is to provide information to facilitate the application of bioaugmentation at DoD facilities. Your participation in this review will significantly assist ESTCP in this process and is greatly appreciated. Please forward responses to the undersigned at the ESTCP program office by 1 November 2003.

Yours truly,

Dr. Andrea Leeson

APPENDIX B
BIOAUGMENTATION CASE STUDIES

B1. AEROBIC BIOAUGMENTATION

B1.1 Gilbert-Mosley Site, Wichita, Kansas

Camp Dresser and McKee (CDM) conducted a pilot-scale demonstration employing an aerobic bacterium to treat groundwater contaminated with chlorinated aliphatic hydrocarbons including TCE, dichloroethene (DCE), vinyl chloride (VC), and tetrachloroethene (PCE) at a site in Wichita, Kansas (Bourquin *et al.*, 1997). The site was contaminated by 50 years of industrial activity and the contaminant plume had grown to over 2,000 acres. The city of Wichita investigated the potential for bioaugmentation to remediate the plume at reduced costs over conventional pump-and-treat methodologies.

Burkholderia cepacia PR1₃₀₁ is a bacterial strain that constitutively produces dioxygenase enzyme, even in the absence of an inducer such as toluene or phenol (Munakata-Marr *et al.*, 1996). The organism had been shown to cometabolically degrade CAHs in laboratory tests but was not previously tested in a field situation.

A pilot-scale biobarrier was designed and installed at the Gilbert-Mosley site in Wichita. Oxygen and the microbial culture were continuously injected into the aquifer to form a barrier to degrade contaminants as they passed through the active zone. The concentrations of CAHs were reduced from approximately 500 parts per billion (ppb) to below detection within 24 hours. CDM estimated an initial degradation rate of 94.5 µg/mL/hour.

The positive results from the pilot test led to the decision to proceed to full-scale application. CDM estimated that the cost savings using bioaugmentation in place of a pump-and-treat system at this site would save the city between \$7 and \$10 million. The full-scale system was never completed because the city determined that the plume was being contained by natural barriers and engineered treatment was not necessary.

While this was one of the first field demonstrations of a successful field application of bioaugmentation for aquifer restoration purposes, the amount of information available in the open literature leaves some questions as to the actual effectiveness of the application. Because the site appears to have been sparged and culture fluids were continuously injected, and because no control data were presented, it is not clear that the removal was due to the addition of the culture. In addition, the observation that PCE was degraded aerobically has not been demonstrated elsewhere. Still, the demonstration was declared a success, and in 1997 the American Academy of Environmental Engineers presented CDM an award of excellence for their effort.

B1.2 Industrial Site, Pennsauken, New Jersey

Envirogen, Inc., conducted a field evaluation of bioaugmentation for treating chlorinated solvent contamination using a strain of *Burkholderia cepacia* (Steffan *et al.*, 1999). The test was conducted at an industrial facility where the groundwater was contaminated with 1,000 to 2,500 µg/L of chlorinated solvents, including PCE, TCE, DCE isomers, and vinyl chloride. The aquifer formation was described as heterogeneous, consisting of silty-fine to medium-grade sand interspersed with thin lenses of gray clay. A pilot-scale test system that included both control and test plots was installed in a higher permeability layer confined between two clay lenses. Each

plot was approximately 12-m in length and contained a set of three nested injection wells, three rows of three nested monitoring well clusters, and a recovery well. Two single monitoring wells were installed at each end of the test plot and one additional nested monitoring well was placed between the two plots.

A variant of *Burkholderia cepacia* PR1₃₀₁ was isolated for its adhesion-deficient properties and identified as *B. cepacia* ENV435 (DeFlaun *et al.*, 1999). The culture was grown in the laboratory in 550 liters of basal salts medium by feeding it alternating batches of sucrose and phenol. The culture was transferred into a 1,100-L plastic tank and shipped to the site, then transferred to holding tanks on site for injection.

Two modes of injection were used. For the first injection, the culture was added to groundwater extracted from the end of the test plot, then recirculated through the injection wells at the head of the test plot. The culture was added to achieve approximately 1×10^{11} cells/mL. During the second injection, the culture was injected directly into the monitoring wells under pressure, then the monitoring wells were cleared using pressurized oxygen. During the evaluation, microbial transport, oxygen distribution, and VOC reductions were monitored.

Microbial transport was evaluated during the first phase of injection based on the recovery of colony forming units (CFU's) on plates containing antibiotics against which the injected strain was resistant. The time required to reach the peak of the recovery curve was used to estimate a linear velocity for the cells which was compared to a conservative bromide tracer. The resulting velocities were combined to calculate a ratio showing the relative movement of the cells to the movement of groundwater. The resulting Br:ENV435 ratios were between 1.26 and 1.43, suggesting that the microbes were easily transported. The recovery data was not as promising since the percentage of cells recovered was much less than expected based on the survivability observed during microcosm testing. This suggested that a large number of cells were either attaching to the sediment or were not surviving the *in situ* conditions. A half-life of 1 to 2 days was estimated using first order decay analysis. The unexpected loss of cells led to the second mode of injection, which was an attempt to distribute a sufficient population of ENV435 to degrade the VOCs. It was not possible to monitor the distribution of the cells over distance from the monitoring wells.

During the first injection, the oxygen was depleted rapidly within 2 meters from the injection point. During the second test, pure oxygen was injected into the monitoring points. The DO in the groundwater was raised to 20 mg/L and declined to greater than 2 mg/L over a 3- to 5-day period.

During the first phase of injection, VOC concentrations showed a marked decrease in the test plot compared to the control plot. While VOC concentrations in the injected water varied over time, the concentrations in the test plot were consistently lower than the injected concentrations. Total VOC concentrations dropped from approximately 2,200 µg/L to below 500 µg/L at most monitoring locations with most of the reaction occurring within two meters from the point of injection. The ratio of VOCs degradable by *Burkholderia cepacia* to compounds which this organism cannot degrade (*i.e.*, (TCE+DCE+VC)/(PCE+DCA+TCA)) decreased over time suggesting that biotransformation was primarily responsible for the observed reduction in

concentration rather than abiotic mechanisms. The VOC concentration in the control plot remained relatively constant over the test period.

During the second phase of injection, the concentration of VOCs was reduced to as low as 50 µg/L suggesting that some benefit had resulted from the additional injections of the culture and the injection of oxygen across the treatment zone. As with Phase 1 injection, the ratio of degradable VOCs to nondegradable VOCs decreased over time, which suggests that biodegradation was the predominant removal mechanism.

The results from the above evaluation show the potential for bioaugmentation at this site after site conditions were oxygenated to favor survival of the added bacterium. They also point out some of the problems associated with the distribution of microorganisms in the subsurface. The fact that most of the chlorinated solvent removal occurred within the first 2 meters could have been caused by insufficient oxygen, insufficient concentration and/or dispersion of ENV435, lack of monooxygenase expression by the microbes, or any combination of the three. The second-phase injection, where culture and oxygen were added at discrete points, did not answer these questions. The results observed suggest that a biobarrier or *in situ* biofilter application may be more appropriate than large-scale distribution of this organism within the aquifer.

B1.3 Chemical Manufacturing Facility, Flemington, NJ

In another field-scale demonstration of bioaugmentation, strain ENV435 was injected directly into a bedrock aquifer (Walsh *et al.*, 2000). To facilitate injecting the strain into the aquifer, pneumatic fracturing was used to expand bedrock fractures. Approximately 550 L of a high cell density culture ($\sim 10^{10}$ CFU/ml) of strain ENV435 was injected into the aquifer formation during the fracturing process, and results of plate count analysis demonstrated that the organism was dispersed throughout the aquifer in a radius of about 25 feet from the fracture/injection well. Cell numbers in groundwater collected from monitoring wells were as great as 10^8 CFU/ml almost immediately after injection.

TCE concentrations in the formation rapidly decreased from between 20 to 30 mg/L to less than 5 mg/L within a few days after injection. A decrease in TCE degradation rate with time correlated with decrease in the viable ENV435 population during the same 2-week period. It was estimated that during that study approximately 825 g of TCE were degraded by approximately 46,000 g (wet weight) of ENV435 cells. This corresponds to an apparent transformation capacity (Tc) of about 0.018 mg TCE/mg cells which is greater than that estimated for a toluene-degrading enrichment culture (0.0073), but lower than that reported for a phenol-degrading enrichment culture (0.031) (Chang and Alvarez-Cohen, 1995).

B1.4 Chico Municipal Airport, Chico, California

Researchers from Lawrence Livermore National Laboratory (LLNL) conducted a field test of an *in situ* biofilter employing a methane utilizing bacterium (methanotroph) in a TCE-contaminated aquifer at the Chico Municipal Airport in Chico, California (Duba *et al.*, 1996). The plume at this site is approximately 500 meters wide by 2,000 meters long with a maximum TCE concentration between 1.0 and 1.5 ppm. The water table is at approximately 26 meters bgs. The plume was restricted to the Tuscan formation, which is characterized as a heterogeneous mix

of cobbles and finer-grained materials. The porosity was estimated at 40% with a permeability of $3\mu\text{m}^2$ and a groundwater velocity of 30 cm/day. Aquifer testing and geochemical analyses showed that the site was suitable for application of the methanotrophic bacterium *Methylosinus trichosporium* OB3b.

Methylosinus trichosporium OB3b has been studied extensively in the laboratory and is known to cometabolically degrade TCE when supplied with methane as the primary substrate. Two 1000-L batches of culture were grown in the laboratory in a 1,500-L fermentor, then centrifuged to a paste, bottled, and shipped to the site on ice. The cells were suspended in TCE-free groundwater to a density of approximately 5.4×10^9 cells/mL, and buffer and tracer were added. Approximately 1,800 liters of the suspension were injected into a single well at approximately 3.8 L/min. No primary substrate was added with the injected culture. Immediately following injection of the culture, 400 liters of uncontaminated groundwater was injected into the well to move the culture and distribute it in the aquifer. Groundwater was then extracted from the well at 3.8 L/min for 30 hours, then at 2.0 L/min for the duration of the test.

Groundwater samples were collected from the extraction well and two monitoring wells located approximately 1 meter from the extraction well. The samples were analyzed for TCE concentrations, bacterial enumeration, and tracer concentrations. The results showed that approximately 50% of the injected bacteria were retained by the aquifer and were presumed to have attached to the sediment. Over the first 50 hours, TCE concentrations were reduced by 98% from 425 ppb to less than 10 ppb. The performance gradually decreased with TCE concentrations in the extracted water increasing to background levels after 40 days.

This demonstration showed that the injected culture was able to degrade TCE for a limited period of time. The culture did not appear to survive but it was unclear if this was due to the lack of a primary substrate or inability of the bacterium to compete and persist in the formation. Predation was ruled out based on the relative numbers of protozoans and *M. trichosporium* cells that were recovered in groundwater that was extracted after 39 days. Regardless, the researchers recognized that the sustainability of the performance needed to be extended beyond the 2 days and that heterogeneity in the subsurface would have an impact on the ability to create an *in situ* biofilter.

B.2 ANAEROBIC BIOAUGMENTATION

B2.1 Dover Air Force Base, Dover Delaware

The Remediation Technologies Development Forum (RTDF), a collaboration between federal and industrial partners (www.rtdf.org), evaluated accelerated anaerobic bioremediation and natural attenuation of TCE in groundwater at Dover Air Force Base (AFB) in Delaware. The RTDF constructed more than 1000 microcosms (Lee *et al.*, 2000) using site soil and groundwater amended with various electron donors including: volatile fatty acids (acetate, lactate), alcohols, sugars (including molasses), and complex organics. Although TCE was reduced to *cis*-DCE regardless of the amendment used, conversion past *cis*-DCE to VC and ethene were observed in only a small percentage of microcosms incubated during the course of these studies (up to 500 days), even when methanogenesis was occurring. This shows that *Dehalococcoides* is sparsely distributed at this site because TCE should have been dechlorinated beyond *cis*-DCE in a greater percentage of these microcosms. Thus, we can conclude that microorganisms capable of

converting *cis*-DCE to ethene were either absent, very sparsely distributed, or inactive at this site.

Harkness *et al.*, (1999) demonstrated the need for bioaugmentation using columns filled with soil from the Dover AFB site. TCE was not degraded beyond *cis*-DCE in columns that had been fed only electron donors for up to 200 days. This timeframe should have been sufficient to stimulate the growth and activity of any indigenous *Dehalococcoides*. Injection of a small volume of a culture containing *Dehalococcoides* (the Pinellas culture) into one of the columns stimulated complete dechlorination of *cis*-DCE to ethene within 20 days in that column. The same effect was later observed in a second column injected with the same culture. VC production was transient in both bioaugmented columns, with rapid conversion to ethene. This supports the conclusion that *Dehalococcoides* microorganisms were not initially present in the aquifer material, but were responsible for complete dechlorination after their addition.

This conclusion was supported by the results of a field bioaugmentation demonstration at the site (Ellis *et al.*, 2000). The pilot treatment area was fed lactate for 269 days, during which time TCE was stoichiometrically dechlorinated to *cis*-DCE. VC and ethene were not produced during this interval. Only after the aquifer was amended with the same culture used in the column studies was *cis*-DCE completely reduced to ethene (Ellis *et al.*, 2000). This result demonstrates the value of bioaugmentation when evidence clearly indicates the absence of organisms capable of complete conversion of *cis*-DCE to ethene. Follow-on analysis using molecular probes (Hendrickson *et al.*, 2002) demonstrated that the *Dehalococcoides* were present in the culture used for inoculation was detected only within, and not outside of, the pilot test area (PTA), again indicating the need for, and success of, bioaugmentation. Additional sampling performed 2 and 3 years after the completion of the pilot test detected the continued presence of *Dehalococcoides ethenogenes*-like bacteria within the PTA, but again not in the upgradient background wells. These data indicate that *Dehalococcoides* can survive for long periods in the subsurface, and continue to dechlorinate as long as an anaerobic environment is maintained.

B2.2 Kelly Air Force Base, San Antonio, Texas

Major *et al.* (2002) conducted a demonstration of bioaugmentation for treating dissolved-phase PCE, TCE and *cis*-DCE at Kelly AFB in San Antonio, Texas. Prior to the demonstration, the site groundwater contained about 1 mg/L of PCE and lower amounts of TCE and *cis*-DCE, without any detectable VC or ethene. Analysis with 16S rDNA-based PCR methods did not detect *Dehalococcoides* in any groundwater or sediment samples from the PTA. Laboratory microcosm studies showed that non-bioaugmented treatments containing lactate or methanol resulted in stoichiometric conversion of TCE and *cis*-DCE, without further dechlorination of *cis*-DCE to VC or ethene. Microcosms bioaugmented with KB-1TM and methanol stoichiometrically converted all of the TCE to ethene. The field test consisted of three recirculation plots, two that served as control plots, and one that was bioaugmented with KB-1TM.

The test plot was recirculated for 89 days to equilibrate the system and to conduct the bromide tracer test. From day 90 to day 175, methanol and acetate were added as electron donors to establish reduced conditions and to stimulate reductive dechlorination by the indigenous bacteria. Bioaugmentation with 13L of KB-1TM occurred on day 176. Performance monitoring of the control and test plots showed that in the presence of methanol and acetate, the indigenous bacteria could be stimulated to dechlorinate PCE to *cis*-DCE. However, no dechlorination past

cis-DCE was observed in the control plots for the remainder of the test. In contrast, VC was detected 52 days after bioaugmentation with KB-1™ in the test plot, and by day 318 ethene was the dominant product. Calculated half-lives for degradation were on the order of minutes to hours. 16S rDNA-based PCR methods were used to monitor the migration and growth of KB-1™ culture after injection. Molecular monitoring showed that the culture had completely colonized the 9.1 meter-long aquifer test plot within 115 days after the one-time injection of KB-1™. The two control plots were installed and operated in the same manner as the test plot, but were never amended with KB-1™. In these control plots dechlorination stalled at *cis*-DCE, with no VC observed during 216 days of operation. Molecular analysis confirmed that *Dehalococcoides* was not present in the control plots.

The most conclusive evidence for the need for bioaugmentation at this site was obtained from molecular techniques, which showed that the “fingerprint” of the *Dehalococcoides* species in the KB-1™ culture had spread throughout the bioaugmented test plot, whereas *Dehalococcoides* was not detected in the control plots or outside of the bioaugmented test plot. This study also showed that there were naturally-occurring *Dehalococcoides* present at a geographically-isolated area of Kelly AFB. Interestingly, these *Dehalococcoides* were located in a waste pit that was very clayey, with little to no groundwater movement, and that had received organic waste and chlorinated solvents for decades. This *Dehalococcoides* had a different “fingerprint” than the KB-1™ bioaugmentation culture, and this different signature was not detected in the field pilot plot that was bioaugmented.

B2.3 Bachman Road Residential Wells Site, Oscoda Michigan

Lendvay *et al.* (2003) conducted a field demonstration of the relative performance of bioaugmentation and biostimulation through side-by-side closed-loop, recirculatory remediation test plots at the Bachman Road Residential Wells Site in Michigan. Molecular analysis indicated that indigenous *Dehalococcoides* populations existed at the site, and this population was enriched (the Bachman Road Culture) and used for the bioaugmentation plot. Two test plots (4.6 x 5.5 m) were constructed perpendicular to groundwater flow, separated by one plot of the same size. Each plot consisted of an extraction well, two injection wells, and a series of performance monitoring points. A bromide tracer study was performed to quantify the hydraulics of each test plot, and a design recirculation flow rate of 7 gpm was selected for each plot. Both the biostimulation and bioaugmentation plots received lactate as an electron donor. The bioaugmentation test plot was preconditioned with a lactate (0.5 to 1.0 mM) nutrient feed prior to bioaugmentation. On day 29, 200 L (10⁸ cell/mL) of the Bachman Road Culture was introduced into the bioaugmentation plot.

Relative to the control (biostimulation) plot, bioaugmentation resulted in a significant reduction in the time to achieve complete dechlorination to ethene. Complete dechlorination of PCE to ethene was achieved within 6 weeks after inoculation in the bioaugmentation plot, whereas, after 4 months of operation nearly 76% of the PCE was converted to ethene in the biostimulation plot. Important findings of this work include: (1) dechlorination in the bioaugmentation plot was demonstratively linked to the presence of *Dehalococcoides*; (2) *Dehalococcoides* populations grew (measurable numbers increased) as system operation proceeded; (3) bioaugmentation significantly shortened lag times to the onset of dechlorination;

and (4) biostimulation approaches can achieve complete dechlorination to ethene at sites where certain *Dehalococcoides* populations occur naturally.

B2.4 Industrial Site, Boston, Massachusetts

GeoSyntec and ERM performed a bioaugmentation pilot test at an industrial facility in Boston (Chang *et al.*, 2002; 2003). Spent organic solvents, primarily TCE, were released to unconsolidated soils through a dry well located interior to the main manufacturing building. The TCE is suspected to have traveled along building pilings downward to the basal unit of fractured bedrock. The PTA is located directly downgradient from the dry well. Concentrations of TCE in the PTA range from 30 to 120 mg/L. Due to the proximity to salt water, sulfate and chloride concentrations in shallow bedrock were approximately 400 and 5,500 mg/L, respectively. Pre-design laboratory studies using PCR and 16S rDNA-based methods detected the presence of an indigenous *Dehalococcoides* population. Microcosm studies confirmed that when supplied with an exogenous electron donor, the indigenous microorganisms could be stimulated to convert TCE to ethene. However, compared to microcosms amended with the KB-1™ culture, the rate of ethene production achieved by the native bacteria was much slower, even after a six month incubation period. Based on the results of the laboratory trials, bioaugmentation was selected for the field pilot test.

A recirculatory, forced-gradient pilot test system was designed based on the demonstrated success of achieving effective reagent delivery and maximum mass balances with these types of pilot-scale systems for bioremediation applications at other sites (*e.g.*, Hopkins and McCarty 1995; McCarty *et al.*, 1998; Ellis *et al.*, 2000). The PTA was comprised of an injection well, extraction well, and three monitoring wells. The PTA layout was oriented such that the induced gradient was parallel with the prevailing ambient flow direction and hydraulic gradient to minimize leakage from the PTA. Prior to performing any biological treatments, the hydraulics of the PTA (*i.e.*, flow rates, residence time, capture, mass recovery) were quantified via tracer testing with iodide. The tracer test demonstrated hydraulic connectivity across the PTA, but only 15% of the iodide delivered to the injection well was recovered at the extraction well. These results indicated a high degree of mixing between the recirculation cell and ambient groundwater. Subsequent observation in the vicinity of the Site revealed that dewatering activities at a neighboring property caused periodic 90-degree changes in the hydraulic gradient in the PTA.

The PTA was fed acetate for the first 3 months of operation for the purpose of establishing reducing conditions in the test zone, prior to bioaugmentation. During this preconditioning period, sulfate concentrations and oxidation/ reduction potential (ORP) decreased linearly, and TCE was dechlorinated to *cis*-DCE. Dechlorination did not proceed beyond *cis*-DCE prior to bioaugmentation. During the fourth month of operation (June 2002), the PTA was augmented with KB-1™ and methanol was added as a supplemental electron donor. Acetate addition was discontinued in October 2002 due to site-specific reasons. Bioaugmentation was achieved by transferring 40L of KB-1™ culture from stainless steel culture containers into the injection well. Argon gas was used to displace the culture from the containers and push it into the well. The bioaugmentation culture volume was calculated based upon a design target of 0.01% of the pore volume in the PTA.

TCE and *cis*-DCE have been degraded to below their respective State remediation standards throughout the PTA. Transient accumulations of *cis*-DCE and VC appeared at peak concentrations that were approximately equivalent to the initial micromolar concentration of TCE. Detectable conversion of VC to ethene began in the latter half of 2002, a few months after bioaugmentation. In 2003, production of ethene continued to increase; however, the ethene concentration did not balance the amount of VC loss observed. The cause for this gap is not known, but is likely related, in large part, to dilution of the plume in the PTA as a result of the shifting hydraulic gradients at the Site. Ethene is also not an entirely conservative end product, as it can be further metabolized. Molecular assays (PCR and genetic analyses) of groundwater samples collected from the PTA after bioaugmentation indicated that the density of *Dehalococcoides* populations in the PTA had increased significantly since bioaugmentation.

B2.5 Caldwell Trucking NPL Site, New Jersey

Golder Associates and GeoSyntec are operating a bioaugmentation/biostimulation system to treat a PCE/TCE DNAPL source area in fractured bedrock groundwater at the Caldwell Trucking Superfund Site in New Jersey. The system is treating a source area in fractured basaltic bedrock in a test area measuring approximately 120 feet wide, and 40 feet long. The source area was bioaugmented (February 2001) with the KB-1™ culture, and electron donors (methanol, lactate and acetate) were added periodically in a batch mode via multiple injection wells. Groundwater circulation was not a component of the design. Initially, electron donor was added on a weekly basis. After monitoring results showed relatively slow treatment performance, the frequency of donor addition was increased to a daily basis.

As of Fall 2002, results indicated an order of magnitude decline in PCE/TCE concentrations, with a concomitant increase in the concentration of *cis*-DCE and VC. There is evidence that *cis*-DCE production has peaked and concentrations are starting to decline. TCE concentrations in the well containing the highest TCE concentration (680 mg/L) have declined by 90 percent. Furthermore, the use of molecular probes has demonstrated that the *Dehalococcoides* microorganisms that were injected in the KB-1™ culture have become distributed throughout the test area.

B2.6 Aerojet, Sacramento, California

A field demonstration was initiated to assess TCE dechlorination in a deep aquifer at the Aerojet Superfund site in California (Cox *et al.*, 2000; Cox *et al.*, 2002). Previous laboratory microcosm studies for the Aerojet site had shown that TCE dechlorination consistently stalled at *cis*-DCE, unless bioaugmented with dehalorespiring bacteria. The addition of lactate alone to the PTA groundwater failed to promote significant TCE dechlorination past *cis*-DCE (VC and ethene were not produced). Bioaugmentation of the PTA with KB-1™ immediately accelerated the rate of TCE and *cis*-DCE dechlorination, and VC and ethene production from *cis*-DCE were observed within eight days following bioaugmentation. Within 125 days, the concentrations of TCE (starting from 2 mg/L), *cis*-DCE, 1,1-DCE and VC were below respective MCLs in the PTA. Molecular characterization techniques (16S rRNA screening using PCR) were used to evaluate the presence of *Dehalococcoides*: i) prior to bioaugmentation, to assess the effects of electron donor addition alone; and ii) following bioaugmentation to track the success of KB-1™ addition, and to assess its transport and survival in the PTA groundwater. Initial sample analyses were negative, suggesting that *Dehalococcoides* was not present in the PTA groundwater. A few

days after bioaugmentation, a strong signal representative of the *Dehalococcoides* strain in KB-1™ was detected in the PTA well where KB-1™ was introduced to the aquifer. A final sample round for *Dehalococcoides* was collected 75 days after bioaugmentation. Wells in the PTA, to a distance of 50 feet from the point of introduction, indicated moderate to strong *Dehalococcoides* signal suggesting transport of KB-1™ through the PTA.

B2.7 Carbon Tetrachloride Site, Schoolcraft, Michigan

Dybas *et al.* (1998), conducted a full-scale field demonstration of bioaugmentation in an aquifer contaminated with CT and nitrate. The demonstration evaluated the performance of bioaugmentation in a biocurtain system designed to intercept and treat the downgradient edge of a CT plume (~ 30 ppb) in a sandy water table aquifer. *Pseudomonas stutzeri* KC was selected for the test because of its known ability to degrade CT without producing chloroform (CF). The requirements for CT transformation by strain KC are (1) adequate concentrations of nitrate and electron donor, (2) anoxic denitrifying conditions, (3) iron-limited conditions, and (4) trace levels of copper. In addition, CT transformation by strain KC is optimal at pH ~8. A pilot study performed at the site previously demonstrated that CT transformation (60 to 65% removal efficiency) could be achieved *in situ* through inoculation with strain KC, addition of acetate and phosphate, and pH adjustment (Dybas *et al.*, 1998). NaOH-amended groundwater was used to maintain slightly alkaline conditions (pH>7.6), KC was injected, and acetate was added in weekly pulses to maintain degradative activity. During a period of poor chemical delivery, incomplete degradation of CT to chloroform occurred and chloroform concentrations increased. However, data from downgradient monitoring wells indicated that as long as the appropriate amendments were maintained, bioaugmentation was effective for CT remediation (Dybas *et al.*, 1998). The pilot study also found that CF generation occurred in regions where strain KC activity was low, and uniform CT transformation was not achieved because of inadequate hydraulic control.

The full-scale system was designed using data and design parameters collected from the pilot test, aquifer characterization, laboratory studies, and solute transport modeling. The full-scale bioaugmentation/biocurtain system was installed in a linear array of 15 adjacent injection/extraction wells aligned perpendicular to the natural groundwater flow gradient. Each well alternately served as either an injection or extraction well during different operational phases. The full-scale biocurtain was approximately 15 m long. The primary bioremediation additives used were acetate (electron donor), sodium hydroxide (pH adjustment), phosphate (nutrient), and strain KC. An above-ground chemical addition system was designed to deliver bioremediation amendments on a weekly basis. System performance was assessed in a series of monitoring wells installed upgradient and downgradient of the biocurtain. PCR techniques were applied to track the extent of migration of strain KC downgradient of the biocurtain.

The demonstration was performed in seven primary phases: (1) aquifer characterization and system installation (days 1 - 52); (2) tracer testing with bromide and fluorescein to assess solute transport between delivery wells and downgradient monitoring points (days 53 – 72) ; (3) pre-inoculation mixing and adjustment to pH 8.2 (days 73-116); (4) inoculation and feeding (days 117 – 199); (5) re-inoculation and feeding (days 200-313); (6) feeding with reduced acetate concentrations (days 314 to present); and (7) solid-phase evaluation of contaminants and microbes (days 336-342 and 1006-1013). During a typical delivery event, a combined flow rate

of 150 L/min groundwater was extracted from alternating delivery wells, circulated through the chemical addition/mixing system, and then injected into adjacent delivery wells. On day 117, the biocurtain was inoculated with 18,900 L of strain KC culture ($\sim 10^7$ cfu/mL). Thereafter, the delivery system was operated weekly for a five hour period to deliver bioremediation additives, followed by a 1 hour reversed flow operation to reduce biofouling at the well screen. On days 200 and 201, half of the delivery well gallery was re-inoculated with 37,000 L of strain KC culture ($\sim 10^7$ cfu/mL) to increase the cell density downgradient of the biocurtain.

Sustained and efficient (98%) removal of CT has been observed in the biocurtain system for over 4 years (Dybas *et al.*, 2002). Transient levels of CF and H₂S were observed, but both disappeared when the concentration of acetate in the feed was reduced from 100 to 50 mg/L. Denitrification was stimulated by addition of acetate and strain KC, and nitrate levels were reduced to below drinking water standards at both acetate doses. Cell migration after the first inoculation appeared limited, suggesting that much of the strain KC culture might have been attached to sediments close to the point of injection. Nine days after inoculation, strain KC and tracer were detected 1 m downgradient of the delivery well gallery, indicating that some cells had traveled at least as fast as the advective groundwater velocity. The culture was also detected at a few locations 2 m downgradient of the biocurtain. Subsequent monitoring confirmed that the initial inoculation achieved adequate colonization of the biocurtain area. Fifty-three days after the northern half of the biocurtain was re-inoculated, strain KC was detected at all locations sampled along the entire length of the biocurtain network.

The Schoolcraft project represents the longest sustained successful bioaugmentation application to date. Based on the absence of CF over most of the demonstration, and the apparent colonization and growth of strain KC, it has been concluded that augmentation with strain KC was the principal mechanism for treating CT. However, Dybas *et al.* (2002) acknowledged that indigenous microorganisms may have also contributed significantly to the degradation of CT. Since no control plot was operated during the demonstration, the influence of the indigenous microflora cannot be known for certain. In any case, the project demonstrated the feasibility of pulsed-pumping operation for achieving effective treatment with low volumes and short durations. Except for the weekly 6 hour period of reagent delivery and groundwater recirculation, the biocurtain was operated as a passive treatment system.

B2.8 Launch Complex 34, Kennedy Space Center, FL

Launch Complex 34 (LC-34) is the site of historic releases of TCE, which is present in the subsurface as DNAPL. Up to 40,000 kg of TCE is present in the aquifer below LC34, suggesting that the restoration of groundwater quality through intrinsic remediation processes will require many decades. As part of an ongoing effort to accelerate remediation at LC34, the NASA Small Business Innovation Research (SBIR) program supported a demonstration of enhanced *in situ* bioremediation of TCE DNAPL was initiated in May 2002. Concurrent performance monitoring for the purpose of technology validation was completed by the USEPA SITE program.

Under intrinsic conditions at LC-34, TCE biodegradation results in the accumulation of *cis*-DCE with limited conversion to VC, suggesting that complete degradation is limited by the absence of the appropriate dehalorespiring microorganisms. Molecular characterization of 16S rRNA sequences of the *Dehalococcoides* microorganisms in groundwater at the facility suggest

that these organisms are members of the Cornell sequence subgroup, which are loosely defined as a phenotype which is not capable of complete conversion to ethene.

Beginning in October 2002, groundwater amended with a dilute solution of ethanol was recirculated through a test plot constructed within the DNAPL source area. Prior to ethanol amendment, the concentration of TCE in the recirculated groundwater was 160 mg/L. The addition of this electron donor, at a concentration equivalent to a four-fold stoichiometric excess to that required to reduce all electron acceptor in groundwater (primarily TCE and sulfate), resulted in an increase in TCE biodegradation and significant accumulation of *cis*-DCE and VC. Electron donor addition and groundwater recirculation was continued until February 2003 (107 days). Subsequently, the test plot was bioaugmented with 40 L of KB-1™. After a five month lag period, a rapid increase in dechlorination rates was observed with ethene concentrations in a stoichiometric excess of initial TCE concentrations at some sampling locations, suggesting that biodegradation resulted in enhanced dissolution of TCE DNAPL at the local-scale.

This study confirms earlier laboratory evidence indicating the dechlorinating activity is not inhibited by the high VOC concentrations typically associated with TCE DNAPL source zones. The occurrence of high VOC biodegradation rates with complete conversion to ethene, coupled with the absence of significant methanogenesis, suggests that bioaugmentation may be an effective approach for both enhanced DNAPL removal and/or biocontainment of VOC-impacted groundwater in DNAPL source zones.

B.2.9 Bioaugmentation with SDC-9 at Naval Station Treasure Island.

Three bioaugmentation applications of SDC-9 have been conducted by Shaw at Naval Station Treasure Island in San Francisco, California. The projects were conducted in two location, IRP Site 24 (original source area treatment and expanded plume treatment), and IRP Site 21 (3rd application). The following describes site conditions and remedial activities at each location.

1) Initial Source Area Treatment, IRP Site 24, Building 99

Building 99 at IRP Site 24 had been used as a dry cleaning facility. Discharges of PCE from a former dry cleaning had affected the groundwater directly beneath Building 99. PCE was detected in the source area beneath the building in excess of 20 mg/L. Although suspected, DNAPLs were not detected in the source area. A plume of dissolved chlorinated ethenes in the unconfined aquifer extended from the source area approximately 1,000 feet down hydraulic gradient to San Francisco Bay. The downgradient plume consisted of a mixture of chlorinated ethenes at concentrations in excess of 20 mg/L.

The affected aquifer was unconfined and consisted of unconsolidated sand and silt dredged from San Francisco Bay. The unconfined aquifer was separated into two zone based on hydraulic conductivity. The upper more permeable zone exhibited a hydraulic conductivity of approximately 15 feet/day and extends from the surface to approximately 20 feet below ground surface. The lower zone exhibited a hydraulic conductivity of approximately 5 feet per day and extends from 20 to 35 feet below ground surface. The aquifer overlies unconsolidated lower

permeability Bay Mud sediments. Sulfate was detected in the affected aquifer at concentrations up to 600 mg/L. ambient ORP was approximately 0 to -50 mv.

The initial bioaugmentation treatment was conducted from May 1, 2003 to March 31 2004. The goal of this pilot study was to show the enhanced degradation of chlorinated ethenes using an anaerobic ISB process over a relatively small, high concentration source area under Building 99. The project also evaluated biostimulation with lactate, and lactate with gaseous hydrogen for enhancement of the ISB process. The data were used to provide a sound technical basis for expanding ISB to the extended downgradient plume.

A preliminary investigation confirmed that native *Dehalococcoides* sp (DHC) was present in the groundwater at the site. Although the maximum ambient DHC concentration before bioaugmentation (as determined by qPCR) was approximately 1×10^6 cells/L, the DHC concentrations in most samples were below detection limits (1×10^3 cells/L). Bench-scale tests conducted using site groundwater and sediment indicated that the indigenous population was capable of complete dechlorination, but the rates of dechlorination, especially of VC, were low. Bioaugmentation with SDC-9 substantially increased dechlorination rates, and promoted rapid degradation of VC.

Based on the bench testing, a field application was conducted. Three distinct recirculation loops were established: the first was biostimulation only (injection of sodium lactate and gaseous hydrogen); the second was bioaugmented (sodium lactate and SDC-9); and the third was biostimulated using only sodium lactate. The system was installed inside Building 99 and treated the high concentration source area. Perimeter monitoring wells were placed around the treatment zone to evaluate lateral migration of the contaminants and DHC during groundwater recirculation.

Bioaugmentation consisted of injecting 60 L of a SDC-9 solution containing 10^{11} cells DHC/L, and stopping recirculation once DHC and lactate were distributed throughout the treatment zone. DHC populations increased to $> 5 \times 10^9$ cells/L throughout the bioaugmentation zone within a few months after injection. These elevated DHC concentrations could only be achieved if significant growth of the culture occurred in situ. DHC concentrations decreased only slightly by one year after injection when all of the chlorinated ethenes had been degraded to near or below the detection limit. RNA analysis performed one year after bioaugmentation indicated that viable DHC were still present in the aquifer.

Addition of SDC-9 substantially reduced the remediation time relative to the biostimulation treatments. Substantial accumulation of VC occurred in the biostimulation treatment loops, but VC did not accumulate in the SDC-9 bioaugmentation zone. Furthermore, the SDC-9 culture appeared to more effectively utilize available hydrogen for dechlorination than the indigenous culture as indicated by substantially lower methane production in the bioaugmentation loop than in the biostimulation loops.

2) Expanded Treatment – Downgradient Plume.

Based on the success of the Building 99 source area project, the system was expanded to treat the 1000-foot long downgradient plume. The downgradient dissolved chlorinated ethene

plume extended from Building 99 and directly under Building 96. Building 96 is approximately 300 feet long and is actively used for storage and dispersion of children's toys by the San Francisco Fire Department. The plume then extends another 700 feet, across a street, under active tennis courts and to San Francisco Bay. Hydrogeologic conditions in this portion of the plume were similar to that of the source area. Total chlorinated ethene concentrations were slightly less than in the source area and consisted of approximately 50% daughter products; however, very little ethene was detected in the downgradient plume.

The expanded treatment system consisted of two substrate and organism delivery systems including a direct push biobarrier system and a groundwater recirculation system. The biobarrier system was installed around the 100 µg/L contour of the total chlorinated ethene plume prior to the startup of the groundwater recirculation system. The purpose of the biobarrier system was to emplace a passive biologically active treatment zone around the downgradient plume to ensure untreated chlorinated ethene containing ground water would not be transported outside the treatment zone during recirculation. The biobarrier system consisted of 108 direct push well points comprised of 1-inch diameter well screens with 20 feet of screen installed to a depth of 30 feet below ground surface. Upon installation, SDC-9-containing groundwater was extracted from the injection wells in the Building 99 treatment area. DHC concentrations in the groundwater were $>1 \times 10^9$ cells/L, and RNA verified the viability of the DHC. The extracted water containing SDC-9 was amended with sodium lactate and injected under pressure into each of the biobarrier wells.

The groundwater recirculation system consisted of 19 injection wells and 26 extraction wells. Unlike the Building 99 treatment in which recirculation loops were established, this extraction-injection well layout was designed to distribute substrate radially away from the injection wells thereby reducing the total number of wells needed and providing better distribution of the substrate. Lactic acid, rather than sodium lactate, was selected as a substrate to reduce the overall substrate cost and because the aquifer was sufficiently buffered to prevent significant reduction in aquifer pH. Based on the results of the bench testing, gaseous hydrogen was incorporated into the recirculation system to further enhance biodegradation. An innovative gas distribution system consisting of gas permeable steel piping inserted into the recirculation line was used to deliver the hydrogen gas at a volume below its solubility. The high flow rate of the recirculation water sheared the hydrogen as it permeated the steel tubing resulting in the production of hydrogen microbubbles.

Bioaugmentation was conducted using a SDC-9 culture containing 1×10^{11} DHC cells/L. Rather than inject the culture into the recirculation line, as was done during the Building 99 project, the culture was delivered directly into each injection well prior to beginning recirculation to ensure rapid and complete distribution of the culture through the treatment area. Prior to bioaugmentation, groundwater redox conditions in each injection well were reduced by addition of sodium lactate solution and to ensure DHC viability after injection.

The recirculation system was started in May, 2005. Groundwater monitoring has been conducted to evaluate the distribution of substrate throughout the treatability study area. Alkalinity was determined during the Building 99 project to be a cost-effective surrogate to VOA analysis when evaluating substrate distribution. Groundwater modeling, conducted as part of the system design, predicted substrate would be effectively distributed throughout the treatment zone

in approximately 60 days. Based on the substrate distribution analysis, the recirculation system was stopped in August, 2005 and performance monitoring was initiated.

Results achieved to date indicate that the extraction of a previously injected SDC-9 culture and reapplication for bioaugmentation appears to be an effective remediation approach. The groundwater recirculation system effectively distributed substrate and organisms throughout the treatability study area, and lactic acid was as effective as sodium lactate in establishing reducing conditions without significantly depressing the aquifer pH. Preliminary results suggest chlorinated ethene biodegradation rates in the treatment zones are similar to those achieved at the Building 99 location.

3) Expanded Treatment, IRP Site 21

A third bioaugmentation project is currently underway at Naval Station Treasure Island IRP Site 21. Hydrogeologic conditions at Site 21 are similar to those at Site 24, but chlorinated ethene concentrations are approximately an order of magnitude lower at Site 21 than at Site 24. The maximum concentration of TCE is approximately 2 mg/L. Furthermore, the plume is substantially smaller, and extends approximately 200 feet from the low concentration point source area to San Francisco Bay. The Site 21 treatment covers the entire affected plume.

Because of the small area at Site 21 a more passive remedial system was evaluated. The treatment system consists of an installation of a permeable biotic-abiotic reactive barrier (PRB) on the downgradient boundary of the plume, and the installation of a direct injection system for biological treatment of the dissolved chlorinated ethene plume. The biological treatment process includes bioaugmentation and biostimulation.

The permeable reactive barrier (PRB) was installed on the downgradient (San Francisco Bay) boundary of the plume to prevent potential migration of contaminants into San Francisco Bay during the injection process, and to provide a continual barrier to contaminant migration. The barrier was constructed by direct push injection of EHC (Adventus), a proprietary blend of zero valent iron and an organic substrate. The installed PRB causes both biotic and abiotic treatment of the chlorinated ethenes. Highly reducing conditions were rapidly established in the PRB area upon injection of the EHC. Ongoing monitoring will evaluate the effectiveness of the PRB over time.

The Site 21 biological treatment system consists of 49 direct-push injection points distributed approximately 20 feet on center throughout the plume. The injection points consist of 1-inch diameter well points screened at intervals to provide substrate to the contaminated zones. Both sodium lactate and lactic acid are being utilized as substrate. Because unbuffered aerobic potable water is used for injection of the substrate, sodium lactate and a small amount of SDC-9 containing water was added to the potable water to establish reducing conditions in the mix water prior to injection.

After initial substrate injection, 60 L of SDC-9 containing 10^{11} cells/L DHC was distributed evenly to among the 49 injection points. Immediately after bioaugmentation a lactic acid-sodium lactate solution was injected into each of the augmented injection points. Because the prior investigations at the base showed that the addition of gaseous hydrogen reduced substrate cost

and increased degradation rates, several of the injection points were converted into gas sparge points and small amounts of gaseous hydrogen are being sparged into each of the converted points on a regular basis.

B.2.10 Pueblo Chemical Depot, CO

Regenesis provided a table summarizing 27 applications of their BioDechlor INOCULUM™ (below). Many of these sites have been discussed in presentations and papers, and these papers were also provided. The following detailed case history is abstracted from the project described in Zacharias et al (2005).

BDI was injected into the subsurface at Solid Waste Management Unit (SWMU) 58, within the South Central Terrace (SCT) of the Pueblo Chemical Depot. The aquifer consists of alluvial and colluvial deposits that unconformably overlie the Pierre Shale bedrock. Much of the alluvium in SWMU 58 is described as clay, clayey sand, and silt.

Historic sampling has shown that contamination does not extend past the creek bed and is contained to the east and west by areas of unsaturated alluvium that flank both sides of the plume. Contamination at SWMU 58 consists of a cDCE plume that remains as a result of the incomplete natural degradation of TCE in the previously excavated source area. Hydraulic conductivities range from 7.2-60 ft/day. Historic dissolved oxygen (DO) and oxidation-reduction potential (ORP) measurements indicate a natural anaerobic tendency in the aquifer. The depth to groundwater varies seasonally and can fluctuate up to 5 feet. The groundwater within the SCT flows in a general southwesterly direction and has been historically high in sulfate and nitrate.

The BDI pilot study consisted of 75 injection points installed in a series of nine rows perpendicular to the groundwater flow direction. HRC-primer (red) was injected into three of the rows. HRC (blue) and BDI (green) were injected into the remaining six rows in alternating points. The direct push subsurface injection probes were advanced to the alluvium/bedrock (aquitard) interface approximately 15 feet (ft) below ground surface. Prior to injection activities, the saturated alluvium thickness was measured at 8 ft. BDI was injected into the saturated alluvium at 0.19 liters/foot (L/ft), HRC was injected at 20 pounds/foot (lbs/ft), and HRC-primer was injected at 8 lbs/ft. The HRC injection depth was decreased on the western portion of the pilot study area due to the decrease in thickness of saturated alluvium. The decrease in saturated alluvium is associated with shallow Pierre Shale bedrock along the upper bank of a paleochannel.

Six monitoring wells were sampled at SWMU 58 to assess the pilot study. CE01, an upgradient well, received microbial analysis only during a one-time event to determine unaugmented levels of naturally occurring bacteria. Groundwater was collected from the site using bailer methods during the initial, 1-, and 3-month sampling events. Low-flow micropurge methods were used during the 6-month and 9-month sampling events. The sampling method was changed to increase sampling sensitivity of dissolved gases and chlorinated volatile organic compounds (CVOCs). Bio-traps, or small sampling devices suspended in monitoring wells to collect representative sample of the microbial community over time.

Initial sampling done in February 2004 indicated non-detect (ND) DHC levels at one well and low DHC counts at another. The original bio-traps were later analyzed for Methanogens (MGN) and total bacteria (eBAC) after high methane concentrations were discovered during the pilot study. Results showed that MGN levels were extremely prior to the injection of HRC, indicating that aquifer conditions were strongly methanogenic prior to the HRC and BDI application.

The December 2004 microbial analysis of an upgradient monitoring well showed high levels of eBAC, sulfate- and iron-reducing bacteria (SRB/IRB), and MGN. The *bvcA* gene (vinyl chloride reductase) was ND and DHC levels were very low. This is to be expected, as the site is naturally low in DHC, and upgradient wells should not have been affected by the BDI and HRC application. Real-time qPCR testing also revealed notable changes in the microbial communities during this study, and the eBAC results indicated that biomass remained fairly stable over the study period at ~10⁶-7 cells/bead for all locations. SRBs/IRBs increased over time at all locations.

Decreases in eBAC, *bvcA*, and MGN levels and increases in SRB/IRB and DHC levels were observed downgradient of the treatment area during the pilot study. The *bvcA* gene was ND at CE02, located downgradient of the treatment area, during this time period. Although movement of DHC has been observed on some sites, BAV1 is a sessile organism that lives on the surface of soil particles. Therefore it is not surprising that increases in the BAV1 population were not observed in downgradient monitoring wells.

Six groundwater monitoring wells were sampled and analyzed for CVOCs, including TCE, cDCE, tDCE, and VC, prior to injection activities and 1 month, 3 months, 6 months, and 9 months after injection activities. Most of the trends discussed below are based upon the more recent sampling events. Additional sampling events are expected to confirm these trends.

In upgradient wells, all CVOC concentrations increased during the pilot study. In the treatment area, TCE and cDCE have decreased, with a corresponding increase in VC throughout the pilot study. A slight decline in tDCE is shown in the 9-month data after concentrations steadily increased during the 6-month sampling event.

None of the wells had significant nitrate, nitrite, or sulfide concentrations throughout the pilot study. TOC levels decreased in the most recent sampling event in each of the monitoring wells sampled, and like the decrease in fermenting organisms, this can likely be attributed to the depletion of HRC-primer, which is a quickly-fermentable carbon source designed to remove large amounts of competing electron acceptors from the subsurface to allow the H₂ released slowly by HRC to be used by reductive dechlorinators for chlorinated solvent degradation. The typical longevity of the HRC-primer applied at the site is estimated to be approximately 3-4 months, at which time the amount of carbon delivered to the system should have declined along with the concentration of TOC.

Sulfate concentrations generally increased in all wells. Low levels of ethane and ethene were detected in all wells except well CM1. Although concentrations of ethane and ethene in

CM1 decreased from 6th month to the 9th month, it is interesting to note that CM1 also has the highest levels of DHC observed at the site. Methane concentrations in well CP7 decreased during the pilot study, while the methane concentrations in CM1 and LFMW08 peaked after 6 months and decreased by 9 months. This data correlates with the qPCR data, as methanogen levels decreased in each of these wells by several orders of magnitude from the 6-month to the 9-month sampling event. CP8 displayed an increase in methane concentration from the 6-month sampling event to the 9-month sampling event. CP7 methane concentrations decreased from the 6th month to the 9th month.

The high loading rate of HRC-primer and HRC, injected into the saturated alluvium, increased methanogenic activity in some of the wells in the existing methanogenic aquifer. It has been shown that methanogenic activity increases in proportion to higher H₂ concentrations (Hemond and Fechner, 1994). After H₂ levels are reduced to a “threshold” concentration, methanogenesis becomes less favorable (Wright and Cox, 2004). Most of the wells have shown reduction of TCE and DCE to VC under methanogenic conditions. In wells where methanogens and/or methane concentrations decreased (CM1, LFMW08, and CP7) VC has begun, per the last sampling round, to decrease and the DHC population has begun to increase. This observation may demonstrate the ability of DHC to compete with methanogens at lower H₂ levels in areas where existing methanogenic conditions exist.

VC levels remained relatively constant in areas where methanogens still dominate and where methane levels continue to increase. DHC populations in these areas have decreased with increasing methanogen populations. Methanogens easily dominate DHC when H₂ levels are high and disrupt the reductive dechlorination process. Sulfate-reducing bacteria have not yet shown to hinder the remediation of VC in this study, despite previous studies that have resulted in the inhibition of VC remediation.

In fact, a lot of the ‘conventional wisdom’ that dictates sulfate inhibits reductive dechlorination is being challenged. The problem is more related to the production of toxic sulfide as the by-product of sulfate reduction. This can be “neutralized” by several factors including the fugacity of sulfide gases and the formation of metal sulfides. The concept that sulfate reduction “captures” all available electrons as a means of inhibition is spurious. As methane and sulfate concentrations continue to dwindle, VC concentrations are expected to decrease.

Bioaugmentation served as an important supplementary technology on this project. The data indicate the added Dhc survived and aided in contaminant reduction. Further, the use of the diagnostic tools as described was important in site management.

APPLICATIONS OF BDI AT 27 SITES

Site No.	Site Name	BDI Injection Date(s)	Amount of BDI applied (L)
1	California Site #1 ^{1,2}	Aug 2003, March 2005	103
2	Michigan Site #1	August 2003	56
3	Ohio Site #1	Dec 2003, June 2004	90
4	California Site #2	April 2004	23
5	Ohio Site #2 ^{3,5}	May 2004, May 2005	28
6	Michigan Site #2	May 2004	8
7	Florida Site #1	May 2004	25
8	South Dakota Site #1	May 2004	15
9	Florida Site #2	May 2004	7
10	Utah Site #1	May 2004	60
11	Kansas Site #1	May 2004	50
12	Colorado Site #1 ^{4,5}	June 2004	41
13	Michigan Site #3	June 2004	4
14	Florida Site #3	June 2004	45
15	Georgia Site #1	September 2004	8
16	Rhode Island Site #1	October 2004	36
17	Oregon Site #1	October 2004	18
18	Michigan Site #4	October 2004	25
19	South Carolina Site #1	December 2004	16
20	South Carolina Site #2	December 2004	560
21	New York Site #1	January 2005	30
22	Florida Site #4	January 2005	112
23	California Site #3	April 2005	256
24	New York Site #2	June 2005	60
25	Florida Site #5	June 2005	13
26	California Site #4	June 2005	4
27	New Jersey Site #1	July 2005	125

The references cited above have been provided as pdf files below, to provide further case study information on Regenes bioaugmentation experiences.



¹ Sharma, P.K. et al. Bioaugmentation Pilot Test to Treat cis-1,2-Dichloroethene in Groundwater, *Proceedings of the Fourth International Conference on Remediation of Chlorinated and Recalcitrant Compounds*. (2004).

² Sharma, P.K. et al. Bioaugmentation after a Stalled Biostimulation Application, *Proceedings of the Eighth International In Situ and On-Site Bioremediation Symposium*. (2005).

³ Wright, W.W. et al. Bioaugmentation and Biostimulation of Recalcitrant Intermediate Chlorinated Compounds, *Proceedings of the Eighth International In Situ and On-Site Bioremediation Symposium*. (2005).

⁴ Zacharias, H.N. et al. CVOC Remediation Using Bio-Dechlor INOCULUM and Diagnostics in a Methanogenic Aquifer, *Proceedings of the Eighth International In Situ and On-Site Bioremediation Symposium*. (2005).

⁵ Ritalahti, K.M. et al. Bioaugmentation for Chlorinated Ethene Detoxification. *Industrial Biotechnology*. Summer 2005, 114-118.